Biology and Chemical Ecology of *Spongospora subterranea* During Resting Spore Germination

Towards a germinate/exterminate control approach for *Spongospora* diseases of potato

by

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BSc in Agriculture, Major in Plant Pathology

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Doctor of Philosophy

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Statements and Declarations

Statement of Originality

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Statement of Co-Authorship and Contribution

The following people and institutions contributed to the publication of work undertaken as part of this thesis:

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Paper 2: Balendres MA, Tegg RS, and Wilson CR. 2016. Resting spore dormancy and infectivity characteristics of the potato powdery scab pathogen *Spongospora subterranea*. Journal of Phytopathology, Volume 165, Issue 5, pages 323-330 (Chapter 3).

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Balendres MA, Tegg RS and Wilson CR. 2014. Somaclonal selection for enhanced resistance to *Spongospora subterranea* tuber and root infection in potato and studies on zoospore dormancy, release and chemotaxis. The 2nd International Powdery Scab Workshop. Pretoria, South Africa. July 28-August 1, 2014.

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Abstract

Resting spores are important for the spread and survival of *Spongospora subterranea*, the causative agent of potato powdery scab and root disease. However, resting spores must germinate and release zoospores (the infective agents) to cause infection. Understanding of the germination process and factors is, therefore, important. The knowledge can potentially have implications for resting spore-inoculum management and *Spongospora* disease control. The biology and ecology of *S. subterranea* during resting spore germination is not well understood. Knowledge of factors influencing resting spore germination have been limited.

This thesis studied the properties of *S. subterranea* resting spores and investigated some chemical factors stimulating resting spore germination. The role of germination-chemical stimulants was also examined. This study used a combination of tomato-plant and zoospore *in vitro* bioassays, light microscopy, a targeted hydrophilic interaction ultra-high performance liquid chromatography–mass spectrometry metabolomic approach, and a greenhouse chemical-soil treatment study.

Some of *S. subterranea* resting spores exhibited dormant spore characteristics, which required specific stimuli to germinate. Although a proportion retains constitutive dormancy characteristics, chemical germination-stimulants were found in potato root exudates and in Hoagland's solution. The low-molecular weight organic amino acids – tyramine and L-glutamine – and sugars – cellobiose and L-rhamnose – compounds stimulated resting spore germination at 0.1 mg/ml solution. The release of these compounds in potato roots were influenced by the plant's physiology and growth conditions. Hoagland's solution contains Iron-EDTA, which stimulated resting spore germination. Germination stimulant Fe-EDTA, in Hoagland's solution, in the presence a susceptible host plant, enhanced root infection. However, Fe-EDTA and Hoagland's solution added into *S. subterranea*-infested soil, a month prior to planting, reduced pathogen inoculum DNA levels in the soil.

Further field studies underpinning the use of germination stimulant compounds could lead to a novel, safe and sustainable chemical approach for the management of *S.*

subterranea inoculum in the soil and thus will augment other *Spongospora* disease control measures.

This thesis advances our understanding of *S. subterranea* biology and chemical ecology during resting spore germination. Substantial development is now beginning to be made in the facet of resting spore germination biology and chemical ecology, which are important aspects of *Spongospora* disease epidemiology and disease control development. There remains much to be learned, but the knowledge presented here will encourage additional studies and research efforts.

KEYWORDS: Powdery scab, *Spongospora* root infection, plasmodiophorid, chemical ecology, potato metabolomics, HILIC UHPLC-MS, resting spore germination, spore dormancy.

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To Ma and Pa

Explanatory Note on Thesis Structure

Most chapters have been published as journal articles and therefore some texts will overlap in the introductory and discussion sections. All chapter references have been listed in the bibliography section. The chapters are separated into themes/topics, but the organisation of this thesis was arranged in a manner where it represents an argument of a typical PhD project. The *Plant Pathology* (journal) referencing style has been adopted for all chapters.

List of Tables

The following list contains shortened captions for each of the tables.

List of Figures

The following list contains shortened captions for each of the figures.

Contents

Chapter 2B. Epidemiology of *Spongospora* diseases of potato: progress and challenges

Chapter 3. Resting spore dormancy and infectivity characteristics of the potato powdery scab pathogen *Spongospora subterranea*

I hope this work will take its place alongside the works of others.

Chapter 1. General Introduction

Many microbes live in the soil surrounding the plant roots (Bais *et al.*, 2006). Most are beneficial microbes, yet some, the pathogens, cause diseases (Rovira, 1959, Schroth & Hildebrand, 1964). The losses caused by plant diseases can be devastating and the cost of controlling these diseases can be very expensive (Pinstrup-Andersen, 2001).

From mashed to French fries, potato is a major food eaten by more than a billion people worldwide. The International Potato Center (2013) considers potato as a principal crop that will help feed the increasing human population, alongside wheat, rice and maize. However, this will only happen if potato production is increased and problems resulting in potato crop losses are eliminated or minimised. Among the major challenges faced by growers worldwide are potato diseases. The best illustration of the impact caused by potato diseases is the mid-19th century Irish Famine (Bourke, 1993). Potato late blight, caused by *Phytophthora infestans*, destroyed potato crops in the field. The spores of *P. infestans* are easily dispersed and are very destructive, killing the host-plant tissue (Mizubuti & Fry, 2006). Many people who depended on potato as their main source of food died of starvation and disease, or emigrated. Today, plant diseases still threaten potato production. Not only production is hampered, controlling diseases increases production costs. For example, fertiliser usage, to boost plant health, has increased and application of often costly and sometimes environmentally damaging fungicides is widespread. About $5 billion per year is spent for controlling potato late blight alone (Haldar *et al.*, 2006). Other major tuber, root and foliar diseases (Fiers *et al.*, 2012) also contribute to production and economic losses of potato.

In 1841, Wallroth (1842) described a tuber disease now recognised worldwide as "powdery scab." Powdery scab is caused by the biotrophic soil-borne pathogen *Spongospora subterranea* f. sp. *subterranea* (Wallroth) Lagerheim. Throughout this thesis, the term *S. subterranea* will be used to denote the potato pathogen. The pathogen belongs to the taxonomic group the plasmodiophorid (Karling, 1968). The common features of this group are: infective agents (zoospores) with two flagella of unequal length; resting spores (thick-walled structure) which enclose and protect the primary zoospores; a zoospore infection process involving "Rhor" and "Stachel" structures (Figure 1. 1) which penetrates the plant cell walls (Bulman & Braselton, 2014); and a unique type of nuclear

division, known as "cruciform division" (see Braselton, 1995). Plasmodiophorids are polycyclic pathogens. Successful primary zoospore infection in the roots produces zoosporangia (Ledingham, 1935) that contains secondary zoospores (Kole & Gielink, 1963). Primary zoospore infection also leads to resting spore development. Proliferation of resting spores in root tissue results in root galling and scab lesions on tubers. This pathogen persists for an extended period in the soil as conglomerates of resting spores (Merz & Falloon, 2009). Seed tubers may also harbour the pathogen supplying inoculum that results in infection of progeny crops (Tegg *et al.*, 2015, Tegg *et al.*, 2016).

Resting spores (collectively as sporosorus) of *S. subterranea* are easily dispersed. Resting spores resist unfavourable conditions (Pethybridge, 1913), protecting the zoospores and preventing germination (zoospore release). This germination-preventive mechanism in *S. subterranea*, however, has not been empirically investigated in detail (Chapter 2A).

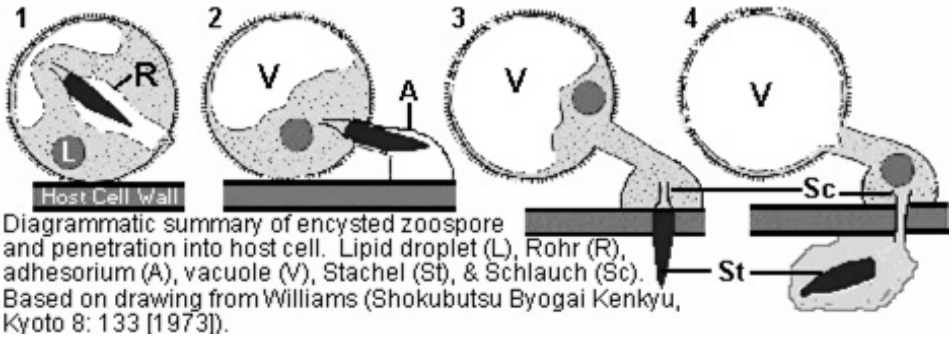

Figure 1. 1. Unique infection process of plasmodiophorids, showing specialised Rohr and Stachel structures. Used with written permission from Paul Williams (University of Wisconsin, USA) and accessed through James Braselton (Ohio University, USA).

1.1. Importance of *Spongospora* disease

Potato (*Solanum tuberosum*), is the world's most cropped vegetable (F.A.O., 2013). More than 300 million tonnes were produced in 2013 with more than half of the tubers produced going to processing factories for chips, fries and other potato by-products.

Potato is important for both human consumption and economic growth. It has nutritional qualities, containing essential vitamins and minerals matching the human's daily dietary needs. Processed potato products are important economic drivers in the potato industry. In Australia, more than half of the total potato tubers produced are processed (HAL, 2011). Recently, this figure rose to about 750,000 tonnes from the total production (Frank Mulcahy, Simplot Australia, *personal communication* 2016). Each year new frozen vegetable products are released with potato as one of the ingredients. Potato's major uses in the processing industry are as chips or fries.

With an estimated loss of A$13 million annually (Wilson, 2016), in Australia alone, *Spongospora* diseases (Wallroth, 1842, Pethybridge, 1913, Ledingham, 1935) are among the most devastating potato diseases (Falloon *et al.*, 2016). *Spongospora* diseases are a major problem in potato fresh and processing industry. Powdery scab diminishes tuber quality, resulting in downgraded tuber-value. Severely infected tubers are discarded (Wale, 2000), affecting the total tuber marketable yield (Gilchrist *et al.*, 2011). For processing, tubers assessed as severely infected with powdery scab may not be marketable. Hence, powdery scab infection can cause 100% losses. Infected tubers that are processed may have a lower value, due to requirements for additional processing and wastage cost (Wilson, 2016). Powdery scab also impedes success in seed-tuber certification standards (U.N.E.C.E., 2006). In Australia, more than 2% of tubers containing one or more powdery scab lesions will lead to failure of certification for seed potatoes. Simplot Australia, one of the world's leaders in processed potato products, had 1500 tonnes of seed in the first half of 2016 rejected for powdery scab. The downgrade from seed price to processing value is about $225/tonne and loss to the industry is about A$ 337,000 (Frank Mulcahy, Simplot Australia, *personal communication* 2016).

Root infection can negatively affect plant growth and development (Lister *et al.*, 2004, Falloon *et al.*, 2004). However, this direct association of root infection with yield loss has been debated (Johnson & Cummings, 2015, Falloon *et al.*, 2016). Infection in both tuber and root by *S. subterranea* has been linked to increase in the activities of other soil-borne potato pathogens (Harrison *et al.*, 1997). In the absence of effective and practical control measures, damage caused by *Spongospora* disease could result in greater disease and further monetary and economic losses. The aim of any plant disease control program is to avoid these losses.

1.2. Current *Spongospora* disease control approaches and future prospect

Spongospora disease control measures can reduce disease incidence and severity (Falloon, 2008). Application of fungicides or the use of putative resistant genotypes/cultivars, for instance, lessens the impact of disease infection (Falloon, 2008, Thangavel *et al.*, 2015). However, "no single method is likely to give complete control of powdery scab, particularly where *S. subterranea* inoculum levels are high on seed tubers or in soil" writes Falloon (2008). Furthermore, the current disease control programs do little in long-term management of resting spore-inoculum in the soil (Chapter 2B). Therefore, there is an abundant source of inoculum for the next cropping season. Exacerbating the problem of high inoculum levels in the soil is the lack of thorough understanding of its survival and germination (Chapter 2A). Only by understanding the processes and factors leading to resting spore germination can we hope to develop effective inoculum and disease management programs – an approach we can learn from the control of potato late blight. More research is needed (Harrison *et al.*, 1997, Merz, 2008, Falloon, 2008, Merz & Falloon, 2009) to improve the current disease control measures or develop and explore new control strategies targeting both the disease and the high levels of inoculum in the soil (Chapter 2A).

Thorough understanding of the disease development processes (pathogenesis) is correlated with improved and effective use of disease control measures. The knowledge helps identify what measure is appropriate to the stage of the pathogen and the disease cycle. Epidemiological understanding also provides information on how to avoid severe infection and evade the factors that favors disease epidemics. For instance, since zoospores are infective when released, any physical or chemical factors that initiate resting spore germination must be avoided. Germination of resting spores (zoospore release) is the most important event in the pathogenesis of *Spongospora* disease (Kole, 1954). Any factors that influence the release of zoospores can affect the subsequent disease outcomes. Knowledge of the factors influencing resting spore survival and germination in *S. subterranea* is limited (Chapter 2A). Consequently, inoculum control programs are often ineffective (Falloon 2008, Chapter 2B). Falloon (2008) emphasised that "expanding knowledge of soil factors affecting survival of *S. subterranea* and development of powdery scab . . . are likely to provide improvements in control of this [*Spongospora*] disease".

1.3. Thesis Objectives

This thesis focuses on expanding the understanding of the biology and chemical ecology of *S. subterranea* during the critical resting spore germination stage. Specifically, this thesis aims to;

1. Examine the biology of *S. subterranea* resting spores in response to environmental stimuli (conditions) (Chapter 3),
2. Investigate the effect of root exudates on resting spore germination (Chapter 4 and Chapter 5), and;
3. Identify compounds that stimulates resting spore germination and to explore the compounds ecological role in managing *S. subterranea* inoculum and controlling *Spongospora* disease (Chapter 6).

The knowledge gained from this study will be critical in selecting appropriate inoculum control measures or in improving some of the current disease management programs. The knowledge will also contribute in developing and exploring other effective inoculum management strategies and disease control measures.

The knowledge gained from this thesis will also encourage further research effort that will lead to greater understanding of the life of *S. subterranea* and the development of *Spongospora* disease.

1.4. Thesis Overview

This thesis is divided into three main sections. The first section provides a comprehensive background of the topic (Chapter 2A and Chapter 2B), followed by the experimental section (Chapter 3, Chapter 4, Chapter 5, and Chapter 6), and concludes with a general discussion (Chapter 7). Figure 1. 2 shows the overview of the experimental chapters.

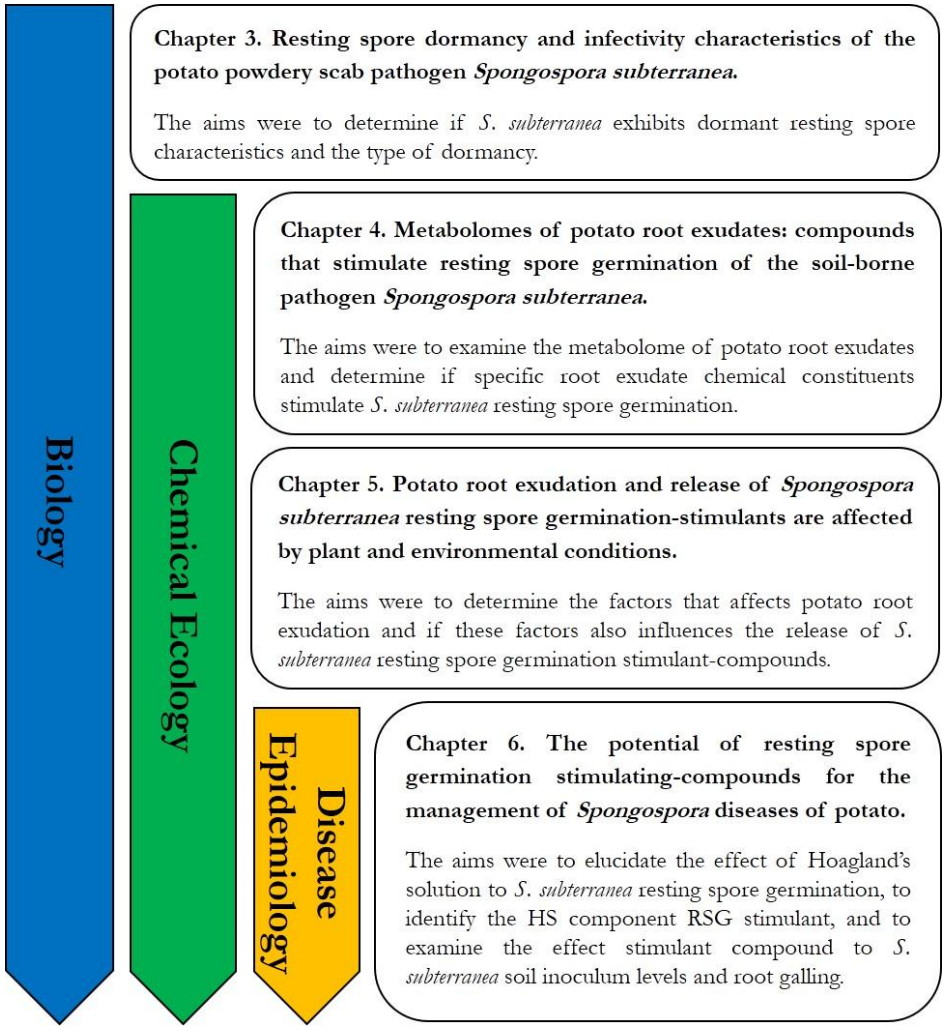

Figure 1. 2. Themes of the experimental chapters and their studied aspect in the *Solanum-Spongospora* pathosystem.

Chapter 2. A. Key events in pathogenesis of *Spongospora* diseases in potato: a review

This chapter has been peer-refereed and published in *Australasian Plant Pathology,* volume 45, No. 3, pp 229-240. The original publication is available at www.springerlink.com. Reproduced with permission of Springer.

2A.1. Abstract

Spongospora subterranea (Wallr.) Lagerheim is the causative agent of powdery scab and root disease of potato. Diseases induced by *S. subterranea* causes substantial economic losses to the global potato industry. The process of disease development in the *S. subterranea*-potato pathosystem has long been studied, but critical events prior to infection and disease development remain poorly understood because the subject has received relatively little attention. Nonetheless, new knowledge of host-pathogen-environment interactions has been gained in recent years. This paper provides the current knowledge of the key events that leads to the development of potato diseases caused by *S. subterranea* and highlights future research to address identified knowledge gaps. This will further our understanding of the interactions between *S. subterranea* and its potato host and contribute to improved disease control measures.

Keywords: Spongospora subterranea, host-pathogen interaction, potato, root galling, plasmodiophorids

2A.2. Introduction

The plasmodiophorid pathogen *Spongospora subterranea* causes three distinct diseases in potato: powdery scab on tubers, zoosporangia root infection and root galling (Falloon *et al.*, 2016). Powdery scab, the most widely recognised disease component, was first observed in 1841 in Germany by Wallroth (1842). The pathogen was initially described as *Erysibe subterranea,* but is now recognised as *Spongospora subterranea* (Wallr.) Lagerh. It was formerly assigned as a forma speciales to separate it from the crook root (of watercress) pathogen *S. nasturtii*, but Dick (2001) subsequently separated them into two

distinct species. Recent genetic and phylogeny analysis based on the ITS rDNA and 18S rDNA confirms that the pathogen is distinct from the watercress pathogen (Qu & Christ, 2004). In 1912, Pethybridge noted that galls are formed in *S. subterranea*-infected roots, but made no clear indication of what preceded gall formation. It was Ledingham (1935) who reported that infection of *S. subterranea* in the roots results in the formation of zoosporangia – a structure common to plasmodiophorids.

That the pathogen originates from South America, the ancestral home of its primary Solanaceous hosts (Melhus, 1913, Hawkes & Francisco-Ortega, 1993), was first assumed by Lagerheim (1892) and Lyman and Rogers (1915). Further evidence for this assumption was provided by Gau *et al.* (2013) from their analysis of the DNA sequences of geographically diverse *S. subterranea* collections and the historical worldwide distribution of potato. To date, the global spread (Figure 2.A. 1) of the pathogen continues with recent new national records in Bulgaria (Bobev, 2009), Iran (Norouzian *et al.*, 2010), Latvia (Turka & Bimšteine, 2011) and Sri Lanka (Babu & Merz, 2011), and within country records in the US states of Colorado (Houser & Davidson, 2010) and New Mexico (Mallik & Gudmestad, 2014), and in the Greek island Crete (Vakalounakis *et al.*, 2013). Most recently, the pathogen was detected in the island-nation of Cyprus(Kanetis *et al.*, 2015).

The diseases induced by *S. subterranea* results in substantial economic impacts on potato production globally. Powdery scab can result in significant losses for producers of seed potatoes as diseased crops may fail certification standards. For example, in Australia, seed tuber crops with >2% of tubers with one or more powdery scab lesions will fail certification (VICSPA, 2007, Tegg *et al.*, 2014) resulting in seed crop devaluation and loss of company/grower reputation. Tuber lesions will also substantially downgrade the value of fresh market potatoes (Harrison *et al.*, 1997).

In the processing sector, growers' crops may be devalued or rejected if disease levels in harvested tubers are high and factories may require additional processing steps to remove tuber lesions; hence, increasing wastage and processing costs (Wilson, 2016). Diseased tubers may also exacerbate water loss and tuber rots in storage with other pathogens gaining entry through *S. subterranea* tuber lesions. In the Australian potato processing sector, which utilises more than half of Australia's domestic production (HAL, 2011) losses due to powdery scab were estimated at A$ 13.4 million per annum, or approximately 4% of the gross production value (Wilson, 2016). In addition to such losses

due to powdery scab, recent studies have indicated significant yield impacts following root infection where the disease affects important plant physiological functions (e.g. water and nutrient uptake) and plant productivity (Falloon *et al.*, 2016). To date, management of diseases caused by *S. subterranea* remains a major challenge with no single effective control strategy (Falloon, 2008).

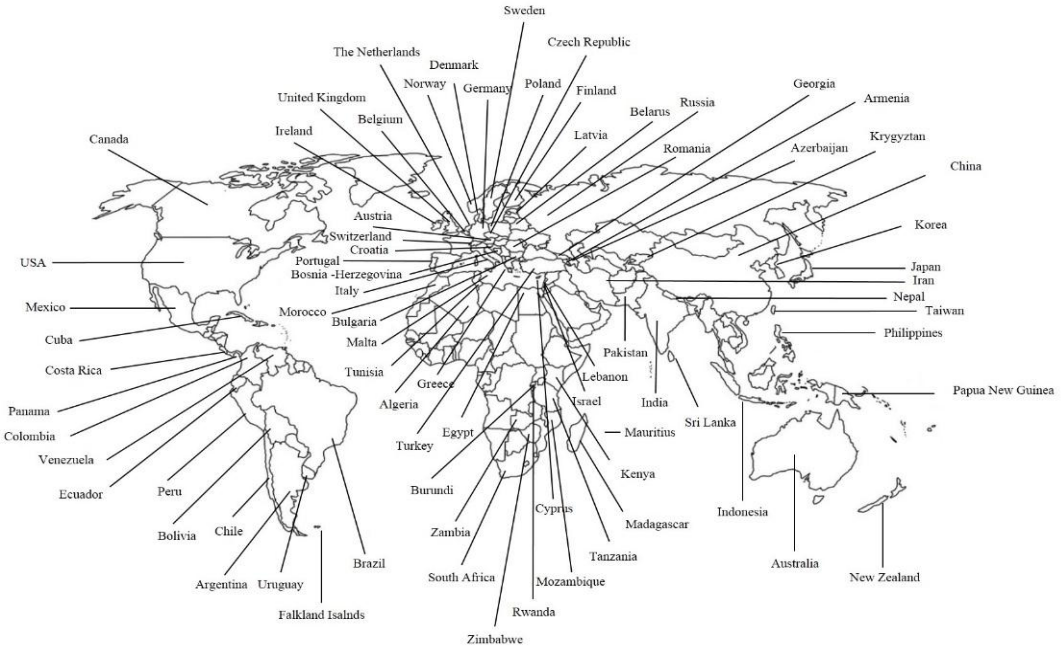

Figure 2A. 1. Global distribution of *Spongospora subterranea*. Pathogen was detected either on potato or tomato. Sources: National records compiled by CABI in association with EPPO (2012) and new reports.

During the last decade, the development of new research tools and techniques has contributed to new knowledge of the interaction between *S. subterranea* and potato. Whilst this new information has improved our understanding of the *Spongospora*-potato pathosystem, studies relating to the events prior to pathogen infection, have received little attention. Hence, pathogen bionomics (interaction of *S. subterranea* to its environment) before it reaches the host is poorly understood and such a lack of clear understanding impedes the development of effective and durable management measures for diseases caused by *S. subterranea*. This paper reviews the current understanding of the critical pre- and post-infection events in the *S. subterranea*-potato pathosystem and examines the physical, biological, and chemical factors involved during these events. This paper

highlights how these factors may influence disease development and how a better knowledge of these factors could be valuable in the development of control measures for potato diseases caused by *S. subterranea.*

2A.3. Pathogen Morphology, Genetics, and Diversity

Spongospora subterranea is an obligate biotroph and as such requires a living host to complete its life cycle (Karling 1968, and Figure 2A. 2). Motile (primary and secondary) zoospores are the means of initiating infection. Each spherical to ovoid zoospore measures on average 4.77$\pm$ 0.15 μm in diameter, with short (4.35$\pm$0.14 μm) and long (13.70$\pm$0.20 μm) flagella attached at the zoospore's posterior (Ledingham, 1935, Kole, 1954). Zoospores have a swimming pattern faster than that of the bacteria commonly associated with inoculum extracts (Merz, 1992). Resting spores provide prolonged survival of *S. subterranea* in soil and infected tubers. Each resting spore measure 4.0 μm in diameter (Osborn, 1911, Lawrence & McKenzie, 1981), although recently Falloon *et al.* (2011) observed resting spores collected from Switzerland that were larger (4.3 μm in diameter). Resting spores are aggregated into sporosori, and these structures measure 18 – 100 μm in diameter (Jones, 1978). Sporosori can each contain approximately 150 to 1,520 resting spores (Falloon *et al.*, 2011). The chemical properties of the substance that binds individual resting spores is unknown. *Spongospora subterranea* shares some similar biological features to those of *S. nasturtii*, a pathogen of watercress. The longevity of viable resting spores in the soil without host plants has not been precisely determined but there are indications that *S. subterranea* resting spores can survive in natural soils for more than 4-5 years. De Boer (2000) detected viable spores buried for almost four years from an artificially inoculated field soil in Australia.

Spongospora subterranea is a phytomyxid species (Bulman *et al.*, 2001) within the plasmodiophorid group, parasitising green plants (Neuhauser *et al.*, 2010). Bulman and Marshall (1998) and Bell *et al.* (1999) initiated genetic characterisation of *S. subterranea* by sequencing the ribosomal transcribed spacer (ITS) DNA regions of the pathogen, which led to the classification of two *S. subterranea* groups (Type I and II). Qu and Christ (2004) found the same group types, but in contrast to earlier characterisation (Bulman and Marshall, 1998), they noted that North American *S. subterranea* DNA samples were Type II. In addition to the existing two types, Osorio *et al.* (2012) proposed a third group (Type

III) based on the ITS rDNA sequences of 127 *S. subterranea* collected from four Colombian provinces. The diversity of South American isolates was further characterised in the work of Gau *et al.* (2013). They found that the more virulent collections (now spread worldwide) were nearly clonal, in contrast to the more diverse collections that are found in South America (Gau *et al.*, 2015). Among the most significant developments in the genetics of *S. subterranea,* to date, is the complete sequencing of the pathogens mitochondrial (mtDNA) genome (Gutiérrez *et al.*, 2014). The mtDNA sequence of *S. subterranea* is the second complete mtDNA genome sequence of a cercozoan and the first of a plasmodiophorid. This sequence will be useful to further validate phylogenetic relationships of plasmodiophorids. Other new findings include the discovery of detailed non-long term repeat (non-LTR) retrotransposons (Bulman et al. 2011), and additional cDNA sequences (Burki *et al.*, 2010) and RNA sequences of *S. subterranea* (Schwelm et al., 2015).

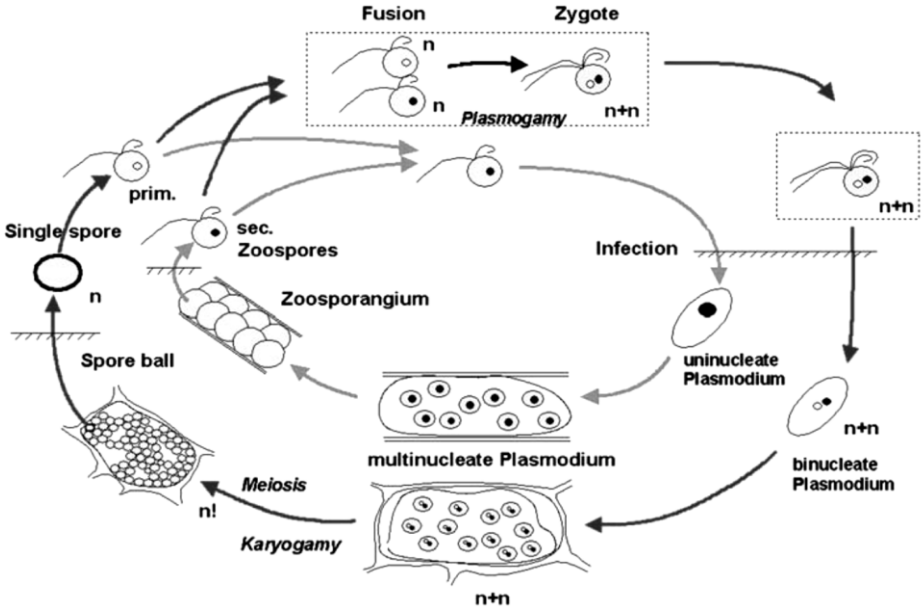

Figure 2A. 2. Tentative life cycle of *Spongospora subterranea* with an asexual phase (inner circle) and possible sexual phase (outer circle) phase (Merz 2008). Reproduced with permission from Dr. U. Merz (ETH Zurich, Switzerland).

2A.4. Pre-Infection Events

Resting spores each possesses a triple wall (Lahert & Kavanagh, 1985) functioning as a protective structure which enables the enclosed zoospores to tolerate unfavourable or extreme environmental condition (Harrison *et al.*, 1997) for survival in the soil for many years. Resting spores of *S. subterranea* will tolerate passage through the digestive canal of farm animals (Pethybridge, 1913, Morse, 1914). Resting spores are, however, sensitive to the soil environment where physical (e.g. heat), biological (e.g. host root exudates), and chemical (e.g. chemical nutrients) factors may stimulate germination (release of short-lived primary zoospores) which if in the absence of a suitable host will perish leading to reduced soil inoculum. Despite their importance on the epidemiology of root infection and powdery scab, knowledge of the specific factors stimulating resting spore germination and zoospore release is limited. Moreover, studies on the factors which influence the movement or attraction of zoospores towards the host (taxis) have not been conducted.

2A.4.1. Zoospore Release

Germination of resting spores within the soil is the critical first step in pathogenesis (Melhus *et al.*, 1916, Dorojkin, 1936, Christ & Weidner, 1988, Merz *et al.*, 2012). Soil environmental factors are important for both resting spore germination and zoospore migration to host roots. The presence of adequate soil moisture, a favourable cool soil temperature, and an external chemical stimulus aid the release of primary zoospores from resting spores (Kole, 1954, Merz, 1989, Harrison *et al.*, 1997, Merz, 1997, Sparrow & Wilson, 2012) although little is known of the nature of the chemical stimulus. Zoospores require free water to germinate and move through the soil solution (Kole, 1954, Braselton, 2001). Zoospore movement towards hosts root increases in the presence of abundant water (Wale, 1987) which is reflected in increased tuber disease incidence in soils with high water retention capacities (Prentice *et al.*, 2007). Irrigated (Taylor & Flett, 1981, Kirkham, 1986, Adams *et al.*, 1987, Jellis *et al.*, 1987, Wale, 1987) and waterlogged conditions (Mol & Ormel, 1946, Hughes, 1980, Anonymous, 1984, Parker, 1984a) are associated with high incidence of powdery scab on tubers. Cooler temperatures of 9-17 °C favour the release of zoospores both in aqueous solutions (Fornier *et al.*, 1996) and in the soil (Van De Graaf *et al.*, 2005, Shah *et al.*, 2012). Higher temperatures in a non-destructive range, however, can stimulate the germination of mature resting spores. For example,

heating sporosori-infested soil for 8 days at 20°C or for 2 days at 40°C increased zoospore release and subsequent powdery scab severity (Kole, 1954). It is unknown if heat can stimulate zoospore release of all sporosorus inoculum from different sources and of different maturities.

Host phytochemical compounds, released as root exudate probably play a role in the stimulus of *S. subterranea* resting spore germination (Harrison *et al.*, 1997, Merz, 2008, Merz & Falloon, 2009) as they do for other soil-borne pathogens (Schroth & Hildebrand, 1964, Nelson, 1990, Suzuki *et al.*, 1992). Merz (1989, 1992, 1997), conducted studies on the behaviour of *S. subterranea* zoospores in relation to host roots and suggested that the root exudates stimulated zoospore release. He observed that the addition of bait-plants resulted in root infection and further suggest that resting spores do not exhibit dormancy with spores germinating in the presence of a host (Merz, 1993). It is, however, unclear if all resting spores germinate when induced by presence of plant roots and perhaps dormancy may still occur in a proportion of the resting spores within a population (staggered dormancy). In the plasmodiophorid *P. brassicae,* both dormant and non-dormant resting spores exist and the mechanism of germination may differ. Non-dormant spores require only a favourable environment whilst dormant spores additionally require an external stimulus for germination (Hata *et al.*, 2002, Ohi *et al.*, 2003, Rashid *et al.*, 2013). In practical terms, as pathogenesis follow a polycyclic pattern of infection, germination of only a proportion of resting spores will be sufficient to initiate root infection and subsequent disease while the presence of spore dormancy will assist in pathogen inoculum longevity in the soil (Ogawa *et al.*, 2001).

Fornier *et al.* (1996) have demonstrated *in vitro* that susceptible-host root exudates stimulate resting spore germination of *S. subterranea* by comparing the rate of zoospore release, using ELISA and microscopy (direct counts), following incubating of sporosori in distilled water and root exudate solutions. They found greater numbers of zoospores in root exudate solution than in distilled water after incubation at 15°C for 5 days. Conversely, in three out of eight experiments, a suppressive effect of tomato root exudates (an alternative host of *S. subterranea*) caused a significant reduction in zoospore number compared to the distilled water control (Fornier *et al.*, 1996). Both Merz (1993) and Fornier *et al.* (1996) agreed that the root exudate factors stimulating resting spore germination (zoospore release) are non-host specific, similar to that of *P. brassicae* (Suzuki *et al.*, 1992, Friberg *et al.*, 2005) and *Pythium* sp. (Nelson, 1990), because non-host plants

may also stimulate zoospore release. It appears that the stimulatory or inhibitory effects of root exudate on *S. subterranea* resting spores depends on the phytochemical stimulant-constituent in the root exudates because this is the only factor that differentiates the root exudates which have been used. The biologically active chemical components of these root exudates are still unknown. In *P. brassicae,* resting spore germination stimulants are heat stable, fairly polar and low-molecular weight compounds (Suzuki *et al.*, 1992, Kowalski, 1996).

Of note, Merz (1997) showed that Hoagland's solution, commonly known as nutrient solution, can stimulate *S. subterranea* zoospore release. Merz (1997) prepared a pulse of zoospore inoculum by incubating sporosori in Hoagland's solution for ten days prior to using the inoculum in a tomato root-bait test. There was no direct observation or quantitation of zoospores in Hoagland's solution before tomato bait-plants were added, but the short time (5 hours) required for observation of zoospore encystment on tomato roots indicated that zoospores may have been released into the solution prior to addition of the bait plants. Fornier *et al.* (1996) and Harrison *et al.* (1997) reported that zoospores are initially released, *in vitro,* 4-5 days after the resting spores are exposed to water or to root exudates which stimulates zoospore release. A nutrient solution similarly stimulates germination of *P. brassicae* resting spores in the absence of its host (Asano *et al.*, 2000, Friberg *et al.*, 2005). Like root exudates, it is possible that only one or a few of the components of the Hoagland's solution has stimulatory effect and thus, requires assessment of individual Hoagland's solution components to identify the compound(s) responsible for stimulating *S. subterranea* zoospores.

2A.4.2. Zoospore Taxis

Upon release, zoospores must travel through the soil water solution to host roots. Zoospores are short-lived (Karling, 1968, Harrison *et al.*, 1997) and can travel only a short distance (Harrison *et al.*, 1997) which means they must be in close proximity to their host and either possess an efficient means to locate their host or be produced in such abundance that random contact occurs frequently. There lacks a precise description on how *S. subterranea* zoospores find their way to their target host. Merz (1997) has shown encystment of zoospores in tomato bait plant roots, but it was unclear whether these

zoospores were attracted to the roots by chemotactic response (movement stimulated by the presence of a chemical gradient) or whether this occurred by chance through close proximity to host roots. Studies on zoosporic fungi (Chi & Sabo, 1978, Zentmeyer, 1961, Dukes & Apple, 1961, Hickman & Ho, 1966, Islam & Tahara, 2001) and nematodes (Zuckerman & Jansson, 1984, Rasmann *et al.*, 2012, Hwang *et al.*, 2015), strongly suggest that motile infective biological agents are attracted to roots via compounds in root exudates. For example, *Phytophthora* spp. zoospores are attracted to certain host isoflavones such as prunetin (Hosseini *et al.*, 2014).

Recognition of the host phytochemicals by zoospores must involve activation of signalling pathways. The zoospores of the oomycete *P. sojae* are attracted to host roots by specific isoflavones (Hua *et al.*, 2008, Hosseini *et al.*, 2014). In this system, the interaction of the attractant chemical with the pathogen zoospore results in a Ca^{2+} influx activating signalling pathways involving heterotrimeric G proteins (Hua *et al.*, 2008). Calcium and calmodium-regulated protein kinases are induced during the zoospore release in *P. sojae*. Calcium channel blockers and protein kinase inhibitors suppressed zoospore release and root encystment (Judelson & Roberts, 2002). Similarly, other studies with oomycetes have shown that swimming patterns may be influenced by Ca^{2+}, which regulates the modulation of flagella (or cilia) beat patterns in other eukaryotic cells (Bloodgood, 1991, Wheeler *et al.*, 2006). Addition of calcium channel blockers (e.g. La^{3+}), calcium chelators (e.g. EGTA), inhibitors of the calcium-binding protein calmodulin (e.g. trifluoperazine) and other compounds that interfere with intracellular calcium levels (e.g. caffeine) alters swimming patterns and removes responsiveness to chemotaxic attractants (Donaldson & Deacon, 1993). Ca^{2+} also plays a central role in autonomous encystment and adhesion of zoosporoic oomycetes (Kong *et al.*, 2010). In *P. brassicae*, increased soil calcium (and increased soil pH) inhibits infection and disease development (Donaldson & Deacon, 1993). High soil calcium levels may reduce the viability of resting spores, and probably interfere with zoospore motility (Donald & Porter, 2009). This is not the case in *S. subterranea* as the addition of soil calcium or increasing soil pH has not been found to affect the disease (Harrison *et al.*, 1997). Nonetheless, the association of ion influx and signalling has not been fully elucidated. Since they play an important role in taxis and encystment of zoospores, understanding the role of ion influx and signalling to *S. subterranea* and disease development could be advantageous. Upon release and after

chemotactic responses, *S. subterranea* zoospores infect host roots within 5 hours (Merz, 1997) or 2 days (Qu & Christ, 2006b).

2A.5. Infection and Disease Development

Once zoospores have reached the plant (root or tuber) surface, the susceptibility of the host becomes a critical criterion for the establishment of infection. Successful zoospore infection of tubers leads to powdery scab whilst in roots lead to root zoosporangial formation (internal symptom) and root galling. The disease cycle is illustrated in Figure 2A. 3.

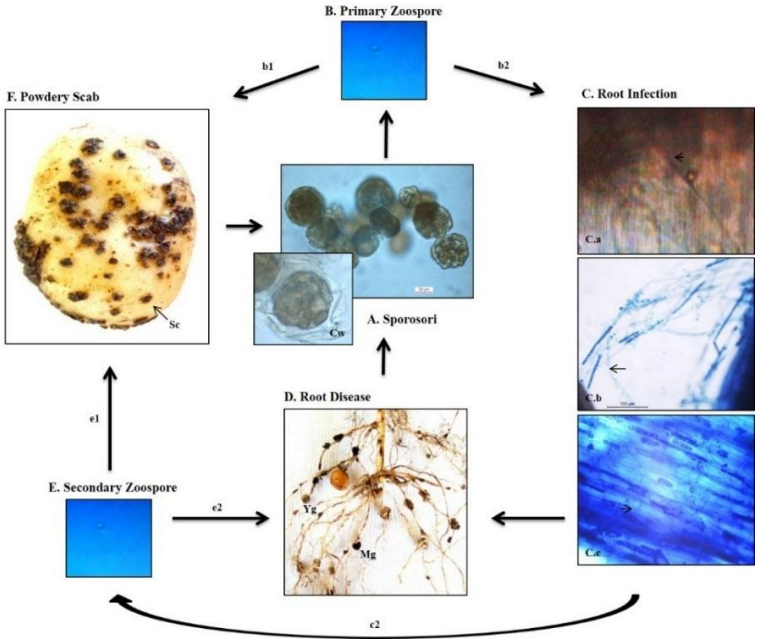

Figure 2A. 3. The *Spongospora* disease cycle. The sporosori (A) release biflagellate heterocont primary zoospores (B) which then infect the tubers (b1) and/or the roots (b2). In the roots (C), zoospore attaches and encysts on roots (C.a), infection results to zoosporangia that are microscopically apparent in root hairs (C.b) and epidermal tissues (C.c). Symptoms of root disease (D) are browning of roots and formation of young creamy-white galls (Yg) – containing many sporosori. When galls mature (Mg) and rot, the sporosori (A) are released back into the soil. A cross section of a gall shows sporosori are wrapped in cell wall compartments (Cw). After discharge from zoosporangia (c2), secondary zoospores (E) infect the tubers (e1) leading to tuber "powdery scab" (F) and can re-infect the roots (e2) causing root diseases (D). Tuber scab lesions (Sc) contain sporosori (A) that are released into the soil when the tuber periderm ruptures.

2A.5.1. Host Recognition and Encystment

Zoospores encyst to the outside of the host and penetrate the cell walls using a specialised 'Rohr' and 'Stachel' structure (Keskin & Fuchs, 1969), common to most plasmodiophorid species, following which the contents of the zoospores enters the cells (Williams, 1970, Kageyama & Asano, 2009). It is understood that the successful zoospore penetration requires some degree of host susceptibility which alongside soil environmental conditions (Merz *et al.*, 2012) and crop agronomic treatments (Shah *et al.*, 2014) dictates the rate of epidemic development (Brierley *et al.*, 2013). In susceptible hosts, the greater the inoculum potential in the soil or on the seed tubers the higher the risk of the disease (Qu & Christ, 2006a, Nakayama *et al.*, 2007, Brierley *et al.*, 2013, Tegg *et al.*, 2015). Binding to the host cell requires specific interaction between pathogen and the host cell wall. In the Oomycetes, host cell surface components are important in enabling induction of encystment to the host tissues. Various plant polymers have been found to induce encystment with some suggestion of specific recognition of certain pathogens (Hardham & Suzaki, 1986). Falloon *et al.* (2016) suggested that host resistance to diseases caused by *S. subterranea* is likely to be influenced by different host genetic, biochemical or morphological factors such as the host cell walls. The cell wall components involved, if there is such, in *S. subterranea* zoospore recognition is not known, nor is it known whether these have a role in cultivar resistance to disease expression.

2A.5.2. Root "Zoosporangia" Infection

Following zoospore encystment and root hair infection a multinucleate plasmodia form within the infected tissue (Braselton, 2001). The plasmodium increases in size and forms into zoosporangia that each contain a nucleus (Kole, 1954, Karling, 1968, Hims & Preece, 1975). Zoosporangia reaches maturity 4-5 days after infection (Merz, 1989). Secondary zoospores are formed from the subsequent division of the nucleus (Lahert & Kavanagh, 1985, Clay & Walsh, 1990).

Zoosporangia is a common sign of infection in alternative hosts of *S. subterranea* (Jones & Harrison, 1969, Jones & Harrison, 1972, De Boer & Theodore, 1997, Andersen *et al.*, 2002, Iftikhar & Ahmad, 2005, Qu & Christ, 2006a, Nitzan *et al.*, 2009, Shah *et al.*, 2010, Arcila Aristizabal *et al.*, 2013). Zoosporangia develops on root hairs and cortical

cells (Lagerheim, 1892). Infections are not restricted to dicotyledonous plants as monocots are also susceptible. There are currently 28 plant families that have been reported susceptible to root "zoosporangia" infection. The Solanaceae (19 species), Poaceae (11 species) and Asteraceae (9 species) families have had the most number of susceptible plant species.

Secondary zoospores released into the soil infect stems (Link & Ramsey, 1932), stolons (Salzmann, 1950., Boyd, 1951) and tubers and can re-infect the roots. Resting spore formation does not occur during the zoosporangial stage in the life cycle, but the increasing zoospore number following re-infection of secondary zoospores in the roots would likely increase the potential of two zoospores fusing into a binucleate zoospore. Root infection caused by binucleate zoospores may lead to sporosori development (Tommerup & Ingram, 1971, Braselton, 2001).

2A.5.3. Root Gall Formation

Galls (Figure 2A. 3) are produced after the pathogen completes the sporogenic stage. Tommerup and Ingram (1971) described how these resting spores are formed in the plasmodiophorid species *P. brassicae*. Accordingly, two uninucleate zoospores fused (now binucleate) and infect the host roots. Following infection, binucleate and multinucleate plasmodia (Braselton, 1992, Braselton, 2001) are formed. Karyogamy within the plasmodium occurs and meiosis follow. Finally, the plasmodial cytoplasm cleaves to give rise to haploid resting spores. To date, however, the final process of the fusing of two nuclei (karyogamy) has not been clearly documented (Braselton, 1995). In *S. subterranea,* Kole (1954) has illustrated quadriflagellate zoospores as possible results of fusions between two biflagellate zoospores. Whilst galling (root hypertrophy) is a possible reaction to the rapid proliferation of sporosori within the infected host roots, such as occurs with *P. brassicae* (Kageyama & Asano, 2009), the presence of galls does not indicate that resting spores are produced. In the United States (Qu & Christ, 2006a), some alternative hosts (e.g. Eastern black nightshade and Penlate Orchard grass) of *S. subterranea* produces galls which do not contain resting spores. Conversely, galls may not indicate that *S. subterranea* has completed its life cycle. In Colombia Arcila Aristizabal *et al.* (2013) claims that some alternative hosts (e.g. Tamarillo, Kikuyu grass) do not produce galls but sporosori are still formed within infected root hairs.

Roots are susceptible to infection throughout the growth cycle of the host plants (Thangavel *et al.*, 2015). As few as 1-10 sporosori g soil^{-1} can initiate severe root infection (Shah *et al.*, 2012). Root infection and subsequent root galling is favoured by soil temperatures of 12-17°C (Van De Graaf *et al.*, 2005) and 11-25°C (Kole, 1954, Van De Graaf *et al.*, 2005, Van De Graaf *et al.*, 2007), respectively . The galls, containing sporosori, are creamy-white to brown. Sporosori are released back into the soil when host roots and galls decay, or when detached from the roots during early harvest.

2A.5.4. Tuber Infection

Early symptoms of powdery scab are small, purple-brown, pimple-like swellings that later increase in size and when matured, rupture the tuber periderm (Osborn, 1911, Lawrence & McKenzie, 1981). Unlike roots, tubers are only susceptible to infection during a defined period early in their growth when they are actively elongating (0-6 weeks after tuber initiation (Hughes, 1980:Van De Graaf, 2007 #29)). Infection is favoured by soil temperatures of 9-17°C but is most severe at 12°C (Hughes, 1980:Van De Graaf, 2005 #31, Van De Graaf *et al.*, 2007, Shah *et al.*, 2012). The shape of the lesions varies, from round to irregular as they coalesce when abundant (Hughes, 1980:Van De Graaf, 2007 #29). Powdery scab symptoms can be confused with those of common scab (caused by *Streptomyces* spp.) but can be distinguished morphologically, by experienced disease inspectors. Detection of abundant sporosori by microscopic examination of lesions will confirm powdery scab (Obidiegwu *et al.*, 2014, Bouchek-Mechiche & Wale, 2014).

2A.5.5. Molecular Host-Pathogen Interaction

Information on host-pathogen interactions at the molecular level is limited. The few available studies have provided insights of the genes involved during the interaction of *S. subterranea* and its hosts. Rodríguez-Fuerte *et al.* (2014) showed that methalothionein, phosphate 2C, and pectin methylesterase inhibitor genes were overexpressed in an infected susceptible *S. phureja* plant, and two different genes (the one associated with α-Galactosidase) were transcribed 1,000 times more than within a non-inoculated plant. The study was carried out using the diploid *S. phureja* and, therefore, warrants a separate analysis of these genes in the tetraploid *S. tuberosum*. Another study (Perla *et al.*, 2014) on

potato cultivars Mesa Russet, Centennial Russet and Russet Nugget, which had shown resistance to powdery scab in repeated glasshouse experiments, found that the level of LOX (Lipoxygenase) gene expression was positively correlated with the russet skinned-cultivars and negatively correlated with the tuber disease severity index. The LOX gene is also upregulated in host plants during the penetration of *P. brassicae* zoospores (Agarwal *et al.*, 2011) and in root-knot nematode feeding sites (Gheysen & Fenoll, 2002). Further studies on metabolic pathways (Bittara, 2013) and gene expression (Gutierrez-Sánchez *et al.*, 2014) have been reported.

2A.6. Future Research

Characterisation of root exudates which stimulate resting spore germination and/or chemotactically attract zoospores to the host roots will provide important knowledge on the biology of the host-pathogen interaction. Characterised compounds may offer novel methods for control of *S. subterranea* infection by reducing soil inoculum levels (stimulating the zoospore release in the absence of the host) and reducing inoculum potential (inhibiting zoospore release). Moreover, if there are compounds that are responsible for chemotactic attraction there might also be compounds that cause repulsion. Compounds that repel zoospores offer a huge potential in the management of diseases caused by *S. subterranea* by disrupting zoospore movement towards the host. This approach has been well documented for nematode control (Chitwood, 2002). Because the characterisation of these compounds are crucial, appropriate and effective analytical techniques will be needed to identify the compounds in the biologically-active root exudates. Previous research has characterised compounds (in biofluids) using a variety of techniques (e.g. inductively coupled plasma atomic emission spectroscopy, (Friberg *et al.*, 2005), but recent improvement in high-throughput analytical technologies provides more sensitive, rapid and selective detection. One such technology includes liquid chromatography coupled to mass spectrometry (LC-MS) and various improvements (such as ultra-high performance LC-MS) which can simultaneously detect more than a hundred low-molecular weight analytes (Gika *et al.*, 2012). Specific compounds or phytochemicals will still need to be individually tested for their biological activity to *S. subterranea* resting spores (stimulation or inhibition) and zoospores (chemoattractant or repellent) and this will require appropriate bioassay methods.

Whilst the potato genome is large, with 844 megabases and as many as 39,031 protein-coding genes (P.G.S.C., 2011), advances in molecular biology techniques coupled to powerful bioinformatics, statistics, data mining and network analysis software have provided tools which can assist in the study of the interaction of *S. subterranean* and potato at molecular level. Identification and characterisation of host genes associated and expressed during infection by *S. subterranea* would facilitate the elucidation of the mechanisms of the compatible host-pathogen interaction, root gall and tuber lesion formation, and identification and characterisation of host defence processes and putative (*R*) genes. Gene sequences already available (Bulman *et al.*, 2011, Gutiérrez *et al.*, 2014) can be further annotated for their structure and functions. Whilst it is expected that multiple (*R*) and susceptible (*r*) genes may be involved, quantitative trait loci (QTL) mapping and association analysis will be essential to characterise the gene(s) controlling resistance. Comparative proteomics have been successful in identifying 46 proteins that were differentially expressed between *P. brassicae* infected *Arabidopsis thaliana* and non-infected roots (Devos *et al.*, 2006, Siemens *et al.*, 2006, Siemens *et al.*, 2009), and hence, a similar technique may be used in *S. subterranea.* Analysis of the roles of differentially expressed proteins will provide insight into pathogenesis processes.

2A.7. Concluding Remarks

This review has focused on the empirical observations of the pre- and post-infection events of *S. subterranea* in potato. It discusses the current understanding on how physical, biological, and chemical factors contributes to pre-infection processes and what changes occur, at the molecular level, during the post-infection stage. Pre-infection events are influenced by soil environmental factors and soil-chemical additives (Chapter 6) but available literature indicates that host factors involving root exudate phytochemicals have important roles which requires further elucidation (Chapter 4). The review has highlighted the knowledge gaps in this pathosystem that requires further elucidation and research. There is much work that needs to be done to clarify the existence of dormancy within resting spores (Chapter 3), to validate the longevity of resting spore survival in the soil in the absence of hosts and to identify the mechanisms of inoculum propagation in alternative host plants. Whilst work on pathogen genetics has progressed, signalling pathways and genetic expression of *S. subterranea* during zoospore release (resting spore

germination) and taxis are still unknown. Genes associated with metabolic pathways active during gall formation and resting spore production also needs to be identified to understand the genetic factors that triggers pathogen development in plants and the nature of spore production particularly in alternative host plants that do not form root galls. The lack of knowledge on the process of host recognition by zoospores is also evident and this impedes our understanding of the mechanisms of resistance by potato cultivars to infection. The identification of host receptor molecules that enable recognition of the pathogen and facilitate encystment would be valuable. Filling these knowledge gaps will significantly improve our understanding of the *Spongospora/Solanum* pathosystem which will contribute to new management options for the potato diseases caused by *S. subterranea.*

Chapter 2B. Epidemiology of *Spongospora* diseases of potato: progress and challenges

2B.1. Abstract

Spongospora subterranea (Wallr.) Lagerheim, a plasmodiophorid soil-borne pathogen, causes powdery scab and root disease of potato. *Spongospora* diseases continue to cause substantial economic losses in the global potato industry. Progress in *Spongospora* disease epidemiology has been slow and some important fundamental epidemiological questions remained unanswered. Consequently, effective disease and inoculum control were partly achieved. Nevertheless, promising approaches to disease, and inoculum, management have surfaced. The lack of complete understanding of the pre-infection activities exacerbated the problems in achieving effective control measures of high levels of soil inoculum. The absence of clear genetic linkage between tuber and root disease resistance also complicated the problems in developing resistant cultivars with commercial merits.

Keywords: powdery scab, Spongospora subterranea, root infection, plasmodiophorid, disease control

2B.2. Introduction

The potato tuber disease powdery scab (Figure 2B. 1A), caused by *Spongospora subterranea*, is one of the challenges faced by growers and the potato industry. Powdery scab contributes to economic losses, affecting the tuber fresh, seed and processing industry (Wilson, 2016). Powdery scab affects the tuber's quality and subsequently the tuber marketability. Infection by *S. subterranea* results in root disease (Figures 2B. 1B and 1C), which may exacerbate the losses by impairing normal plant and root functions. Severe infection may lead to yield reduction (Falloon *et al.*, 2016). Although significant yield impact is debated, for example, no yield loss was observed in a recent field study (Johnson & Cummings, 2015). In Australia, losses due to *Spongospora* disease have been estimated at A$ 13.4 million annually (Wilson, 2016).

Here, development in *Spongospora* disease epidemiology is reviewed. Present and promising *Spongospora* disease control strategies are also outlined.

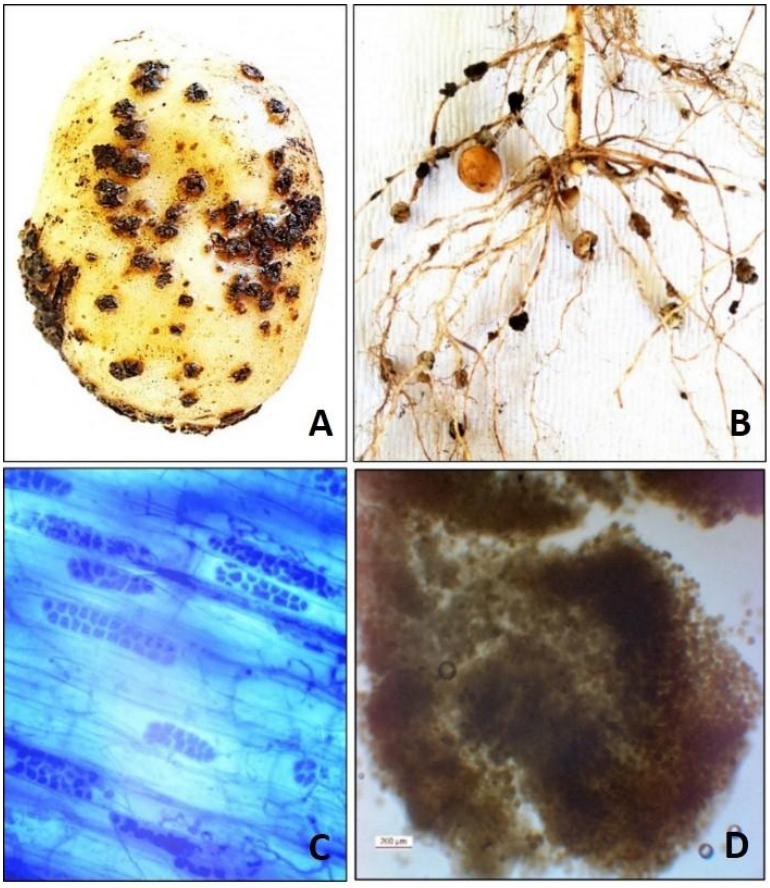

Figure 2B. 1. Powdery scab (A), root galling (B), mass of sporosori (C) and zoosporangia formation within roots (D).

2B.3. Inoculum

2B.3.1. Sources

Spongospora subterranea survives as sporosori (aggregates of resting spores) in the soil in between crops and in seed-tubers during storage. Sporosori are contained in large number in tuber lesions and root galls (Figure 2B. 1D). Each sporosorus consists of an average of 700 resting spores with each resting spore containing a zoospore (Falloon *et al.*, 2011). The primary zoospores, originating from resting spores, upon release find a host, encyst (Keskin & Fuchs, 1969) and the infection leads to zoosporangia formation in root hair and cortical tissue (Ledingham, 1935). Zoosporangia contain secondary zoospores which, when discharged into the soil, infect tubers and reinfect roots (Kole & Gielink, 1963). The cycle continues until the plant reaches maturity. At any stage of the infection cycle, sporosori are formed within root galls and tuber lesions. Two uninucleate

zoospores are believed to form a binucleate zoospore which then encysts on the host (Kole, 1954, Braselton, 2001). The infection leads to the formation of multinucleate plasmodia which later form into resting spores containing uninucleate zoospores. This stage of infection is believed to be a vital step for resting spore production and survival in the absence of a growing host of plasmodiophorid species and was first studied in detail by Tommerup and Ingram (1971). However, Dylewski (1989) and Miller and Dylewski (1983) have indicated that the fusion of zoospores may not be a necessary step in the formation of multinucleate plasmodium. They observed that by manipulating the environment, the infection of a uninucleate (single) zoospore of *Woronina pythii* could develop from sporangial plasmodia (which precedes zoosporangia formation) to sporogenic plasmodia (which precedes resting spore formation). When root galls decay and tuber lesions erupt, resting spores are released (back) into the soil as new primary sources of inoculum. Merz (1993) indicated that *S. subterranea* resting spores are unlikely to exhibit dormancy whilst others suggest otherwise (Harrison *et al.*, 1997, Fornier, 1997). Spore dormancy has long been viewed as an important feature of *S. subterranea* to persist and remain viable in the soil (Harrison *et al.*, 1997, Merz, 2008, Merz & Falloon, 2009), yet few empirical observations have been reported and research underpinning such feature have been lacking.

2B.3.2. Dispersal

Infected seed tubers are the main means of inoculum transfer between fields (Harrison *et al.*, 1997, Merz & Falloon, 2009). Melhus (1913) gave an early historical account of how *S. subterranea,* in potato seed-tubers, was disseminated from Europe to the United States and within North America thereafter. Recently, Gau *et al.* (2013) traced the distribution flow of *S. subterranea* from South America to the rest of the world based on combined information on the pathogen's genetic characteristics and history of potato dissemination. *Spongospora subterranea* was recently detected in the southern Greek island of Crete (Vakalounakis *et al.*, 2013) and in the island-nation of Cyprus (Kanetis *et al.*, 2015). It was pointed out in these reports that the pathogen was most likely transported to these islands via importation of symptomless potato-seed tubers, which still harboured sporosori-inoculum. Tegg *et al.* (2016) demonstrated that symptomless tubers particularly after passing through a seed-tuber grading process can harbour significant inoculum levels

and can cause subsequent infection in progeny crops. *Spongospora subterranea* has spread worldwide in most potato production regions (Chapter 2A).

Farming operations also contribute to plasmodiophorid pathogen inoculum dispersal and introduction to non-infested fields. Irrigation water through irrigation can assist the movement of *Plasmodiophora brassicae* (clubroot of crucifers) inoculum as a result of soil runoff from infested areas to clean paddocks (Datnoff *et al.*, 1984). Farm machinery can also harbour infested soil, which can be transferred to another field during operations (Strelkov *et al.*, 2007). There are no indications that *S. subterranea* resting spores are vectored by insects, but farm animals may assist the transfer of infested soil from one paddock to another through soil adhering to the animal's feet. Wind is generally not considered an important dispersal element, however, it is possible that resting spores exposed on the surface of the soil after land preparation can be blown by wind and spread through the uninfested field. Rennie *et al.* (2015) reported wind blown *P. brassicae* resting spores.

2B.3.3. Survival

Spongospora subterranea resting spore persists in the soil for an extended period. It has a thick three-layered wall (Lahert & Kavanagh, 1985) which protects the zoospore from degradation in the soil. Chapter 2A discussed that this survival may also be in part due to resting spore dormancy. Some field observations have indicated that resting spore can survive for more than four years in the soil. De Boer (2000) found resting spores still viable after almost four years, buried in artificially inoculated soil. In an annual monitoring of major potato pathogens soil DNA in Australia, Sparrow *et al.* (2015) recorded high levels of *S. subterranea* DNA present in soil for eight years after potato cropping. Whilst it is likely that resting spores could survive for more than four to eight years in a natural soil, the question of whether their (resting spores) ability to release zoospores and infect the hosts remain the same or are altered as they age and can be affected by changing soil condition. Such knowledge will be essential for assessing risk of powdery scab and in selecting appropriate control measures.

2B.3.4. Detection and Measurement

Several approaches can detect the presence of *S. subterranea* in plant tissues and in the soil (Table 2B. 1). Traditionally, visual assessments and plant-bait tests have been employed, but with the advent of serology and DNA-based techniques, pathogen detection has become easier, faster and the results were more reliable. The enzyme-linked immuno sorbent assay or ELISA (Harrison *et al.*, 1993, Walsh *et al.*, 1996, Bouchek-Mechiche *et al.*, 2000, Norouzian *et al.*, 2010) and polymerase chain reaction or PCR (Bulman & Marshall, 1998, Bell *et al.*, 1999, Qu & Christ, 2004) techniques have been used for this purpose.

Recently, real-time PCR has been used to quantify *S. subterranea* DNA in tubers (Tegg *et al.*, 2014, Tegg *et al.*, 2015), roots (Hernandez Maldonado *et al.*, 2012, Thangavel *et al.*, 2015) and in soil (Brierley *et al.*, 2009, Shah *et al.*, 2014). Tegg *et al.* (2016) demonstrated the usefulness of PCR in detecting *S. subterranea* on symptomless potato tubers. The study highlighted the importance of pathogen DNA testing to guarantee that seed-tubers do not harbour significant quantities of the pathogen. One critical aspect in pathogen detection and disease evaluation is the method of sample collection that will represent the area or seed lot being evaluated. For example, Tegg *et al.* (2014) found that taking two samples of 100 tubers from the beginning and end of a seedlot or by continuous sampling of ten lots of 20 tubers randomly collected from a seedlot provides comparable levels of disease assessment.

However, Merz and Falloon (2009) suggest that PCR should be coupled to a bioassay (plant-bait) when assessing root infection or associating root infection to soil inoculum. Although PCR is sensitive, the technique is unable to distinguish between viable and non-viable resting spores. A bioassay will confirm infectivity and hence the viability of activated resting spores. Whilst there are several chemical stains (e.g. Evans blue stain, Tetrazolium bromide, Calcofluor stains) that can be used to detect viable spores, none yet have been successfully used for *S. subterranea*. The plant-bait test (Merz, 1989) remains the most rapid, inexpensive, and easy method for assessing viable *S. subterranea* resting spores. Furthermore, although PCR is very useful and generates faster (and robust) results, PCR is less-cost effective for use in the field by farmers.

Table 2B. 1 Approaches and methods used for detecting *Spongospora subterranea*.

Approach	Methods	Some Key References
Visual	Examination of diseased materials relying on apparent scabs (tuber) and galls (root)	Falloon et al. (2003), Genet et al. (2007)
Plant-Bait	Using potato or tomato plantlets to trap zoospores	Flett (1983), Merz (1989)
Microscopy	Detecting sporosori in sample mounted on glass slides and examined using a microscope	Falloon et al. (2011)
	Staining samples to detect the presence of plasmodia and zoosporangia and examining stained samples using a microscope	Ledingham (1934), Merz (1989), Falloon et al. (2011)
	Detection of zoospores	
	By light microscope in solution	Fornier et al. (1996), Harrison et al. (1997)
	By staining	Kole (1954)
	By Scanning Electron Microscope (SEM)	Merz (1992), Merz (1997)
Serological	Identification and quantitation using antibody-based detection method (AgriStrip, ELISA)	Harrison et al. (1993), Walsh et al. (1996), Bouchek-Mechiche et al. (2000), Merz et al. (2012)
Molecular	Polymerase Chain Reaction (PCR) technique using S. subterranea-specific primers and probes for detection and quantitation	Bulman and Marshall (1998), Bell et al. (1999), van de Graaf et al. (2003), Nakayama et al. (2007), Hernandez Maldonado et al. (2012), Brierley et al. (2013) , Merz et al. (2012), Tegg et al. (2015)

2B.3.5. Disease Development

Spongospora subterranea infection cycle follows a polycyclic pattern (Neuhauser *et al.*, 2010). As such the initial inoculum level is of importance to the eventual disease level less than the rate of pathogen re-infection processes. For example, Van De Graaf *et al.* (2005) found no significant differences in percent infection and severity of root infection and powdery scab in potato plants grown with 0, 5, 15, and 50 sporosori per gram of soil. Nakayama *et al.* (2007) and Tegg *et al.* (2015) observed no relationship between sporosori density in the soil and tuber severity, but found a positive relationship between the amount of root infection and tuber severity . In contrast, Qu and Christ (2006a) and Brierley *et al.* (2013) found a significant relationship between soil inoculum and disease incidence in the field. In a glasshouse and shade house study, Shah *et al.* (2012) observed that inoculation with as few as 1-10 sporosori and 25 sporosori per ml soil, respectively, results to severe powdery scab and root galling.

2B.4 Host

2B.4.1. Genotype Variation

Spongospora subterranea primarily infects potato. Although potato genotypes show varying levels of resistance and susceptibility to *Spongospora* diseases, no genotype is immune to infection (Falloon *et al.*, 2003, Merz *et al.*, 2012). Hernandez Maldonado *et al.* (2012) suggests that host resistance may likely have more influence during the zoospongial stage than during the sporosori development in both tuber scab and root galls. However, whether the development of zoosporangia in resistant plants is due to fewer successful zoospore infection or slower zoosporangial development is yet to be examined.

2B.4.2. Plant Age and Growth Condition

The early stage of tuber formation is more susceptible to pathogen infection than at tuber maturity (Van De Graaf *et al.*, 2005). The roots, on the other hand, are susceptible at all plant developmental stages (Thangavel *et al.*, 2015). Root infection progresses with time and is most severe when plants reach maturity (Van De Graaf *et al.*, 2007). Therefore, delaying the onset of infection could reduce the severity of infection at maturity.

2B.4.3. Beyond Potato: Alternative Hosts

Spongospora subterranea infects plant species in 28 families (Table 2B. 2). Some non-potato hosts are also economically important and high-valued crops including tomato, carrot, pea, oats, barley and some important members of the Brassica family such as yellow mustard, rapeseed, turnip and cauliflower. It is possible that these high value crops may be impacted by infection as is the potato (Gilchrist *et al.*, 2011, Falloon *et al.*, 2016), although this has not yet been examined nor reported. Root infection (zoosporangia) in alternative hosts, develops on root hairs and cortical cells and the infection is not restricted to dicotyledonous plants with monocots also susceptible. The Solanaceae (19 species), Poaceae (11 species) and Asteraceae (9 species) families had the most number of susceptible plant species. There are no family-specific symptoms observed. Zoosporangia alone or with root galls (with and without sporosori) had been observed across different families, but symptoms expressed, notably, were country-specific. Zoosporangia were

observed in the United Kingdom (Jones & Harrison, 1969, Jones & Harrison, 1972), Australia (De Boer & Theodore, 1997) and Denmark (Andersen *et al.*, 2002) but in the United States (Qu & Christ, 2006a) and Colombia (Arcila Aristizabal *et al.*, 2013), both zoosporangia and galls (with or without sporosori) are formed in infected roots. In the United States (Qu & Christ, 2006a), some species formed galls without sporosori and the reason for this remains unclear but could be due to plant stress caused by zoosporangia infection and not a reaction to sporosori proliferating within the roots.

In Colombia, Arcila Aristizabal *et al.* (2013) claimed to have observed sporosori in roots without forming zoosporangia and galls. If such phenomena indeed exist, this implies that the absence of galls is not an indicative of an alternative host or bait-plant. However, it could be also due to early sampling or host delayed reaction to infection. It hasn't been clearly indicated if the pathogen's genetic groupings (Type I and II) have a bearing on the observed variation in symptoms. Variations within alternative host's genotypes or cultivars has been proposed (Arcila Aristizabal *et al.*, 2013). Identification of host-plant genes involved during sporosori and gall development would likely assist in understanding the interspecies variations of root infection symptoms caused by *S. subterranea*. Additionally, a standardised method for screening alternative host is essential to ensure uniformity and consistency of experiments – including inoculum source and concentration, plant cultivars, age and growth condition, soil type and temperature, and detection methods – from one country (or assay) to another and to confirm that these plants are indeed alternative hosts of *S. subterranea*. Furthermore, a combination of histological examination and species specific testing (serology and/or PCR) will ensure that the observed infection is caused by *S. subterranea.*

Table 2B. 2. Alternative hosts of *Spongospora subterranea* and reported symptoms on roots.

Plant Family	Scientific Name	Common Name	Origin of Report	Symptoms on Roots
Aizoaceae	*Tetragonia expansa*	Sea spinach/Tetragon	United Kingdom	Z
Alliaceae	*Allium cepa*	Onion	Colombia	Z, S
	Taraxacum officinale	Dandelion	Colombia	Z, S
Amaranthaceae	*Amaranthus retroflexus*	Pigweed	United States	Z
Apiaceae	*Apium graveolens*	Celery	Colombia	Z
	Coriandum sativum	Coriander	Colombia	S
	Daucus carota	Carrot	Colombia	Z, GS
	Petroselinum crispum	Parsley	Colombia	Z, S
Asteraceae	*Ambrosia artemisiifolia*	Common ragweed	United States	Z
	Artemisia vulgaris	Common wormwood	Denmark	Z
	Lapsana communis	Lapsana/Nipplewort	United Kingdom*	Z
	M. inodora (Anthemis arvensis)	Mayweed/Field chamomile	Denmark	Z
	M. discoidea (Chamomilla suaveolens)	Pineapple weed	Denmark	Z
			United Kingdom*	Z
	Senecio oleraceus	Common sow thistle	Colombia	Z, S
	S. vulgaris	Common groundsel	United Kingdom*	Z
	Sonchus arvensis	Field sow/Swine thistle	Denmark	Z
	Tussilago farfara	Coltsfoot	United Kingdom*	Z
Boraginaceae	*Myosotis arvensis*	Field forget-me-not	United Kingdom*	Z
Brassicaceae	*Brassica campestris*	Yellow mustard	United States	Z, GS
	B. napus	Rapeseed	United States	Z
	B.oleracea	Canola/Oilseed Rape	Australia	Z
		Cauliflower	Pakistan	Z
		Rapeseed	Colombia	Z, S
	B.rapa	Turnip	United Kingdom	Z
			Pakistan	Z
			Australia	Z
	Capsella bursa-pastoris	Shepherd's-purse	United Kingdom*	Z
	Raphanus sativus	Radish	United States	Z
			Colombia	Z, S
			Pakistan	Z
	Sinapis arvensis	Field/Wild mustard	United Kingdom*	Z
Caryophyllaceae	*Stellaria media*	Chickweed	United Kingdom	Z
Cyperaceae	*Cyperus esculentus*	Yellow nutsedge	United States	Z
Chenopodiaceae	*Beta vulgaris*	Beetroot/Sugarbeet	United Kingdom	Z
	Chenopodium album	Lambsquarters	Denmark	Z
			United Kingdom	Z
			United States	Z
	C. amaranticolor	Chenopodium	United Kingdom	Z
	C. quinoa	Quinoa	United Kingdom	Z
	Spinacia oleracea	Spinach	United Kingdom	Z
Cucurbitaceae	*Cucumis sativus*	Cucumber	Colombia	S
Fabaceae	*Phaseolus vulgaris*	Common bean	Colombia	S
	Pisum sativum	Pea	Colombia	Z, S
			Pakistan	Z
	Trifolium pratense	Red clover	United Kingdom*	Z
			United States	Z
	T. repens	White clover	United Kingdom	Z
Fumariaceae	*Fumaria officinalis*	Common furmitory	United Kingdom*	Z
Geraniaceae	*Geranium pusillum*	Small geranium	Denmark	Z
Labiatae	*Galeopis tetrahit*	Common hemp-nettle	United Kingdom*	Z
Linaceae	*Linum usitatissinum*	Linola	Australia	Z

Z, zoosporangia only; S, sporosori only; GS, galls with sporosori; G, galls without sporosori.

Table 2B. 2 *Continued*

Papaveraceae	*Papaver rhoeas*	Common poppy	United Kingdom*	Z
Plantaginaceae	*Plantago major*	Broadleaf/Greater plantain	United Kingdom*	Z
Poacaeae	*Arena sativa*	Oat	United States	Z, GS
	Dactylis glomerate	Penlate orchard grass	United States	Z, G
	Hordeum vulgare	Barley	United Kingdom	Z
			Australia	Z
	Lolium multiflorum	Ryegrass	Australia	Z
			United Kingdom	Z
	Pennisetum clandestinum	Kikuyu Grass	Colombia	Z, S
	Pennisetum sp.	Fountaingrasses	Colombia	S
	Phleum pratense	Climax Timothy	United States	Z
	Poa annua	Annual meadow grass	United Kingdom	Z
	Secale cereale	Rye	United States	Z
	Triticum aestivum	Wheat	Australia	Z
	Zea mays	Corn/Maize	Colombia	Z, S
			Pakistan	Z
Polygonaceae	*Fagopyrum esculentum*	Buckwheat	United States	Z
	Polygonum aviculare	Common knotgrass	Denmark	Z
	P. convolvulus (Fallopia convolvulus)	Wild buckwheat	Denmark	Z
	P. nepalense	Nepal persicaria	Colombia	Z, S
	P. persicaria	Redshank	United Kingdom*	Z
	P. segetum	Polygonum segetum	Colombia	Z
	Rumex acetosella	Sheep's/Red/Sour Sorrel	Denmark	Z
			United Kingdom	Z
	R. crispus	Curly/Curled/Yellow Dock	Colombia	S
Ranunculaceae	*Ranunculus acris*	Tall/Giant buttercup	United Kingdom*	Z
Resedaceae	*Reseda lutea*	Wild mignonette	United Kingdom*	Z
Rosaceae	*Rubus glaucus*	Andean Raspberry	Colombia	S
Rubiaceae	*Galium aparine*	Cleavers	Denmark	Z
Solanaceae	*Cyphomandra betacea (Solanum betaceum)*	Tamarillo	Colombia	S
	Datura stramonium	Jimsonweed	United States	Z, G
			Colombia	Z, S
			Pakistan	Z
			United Kingdom	Z
	Lycopersicon esculentum	Tomato	United Kingdom*	Z
			United Kingdom	Z
			United States	Z, GS
			Australia	Z
			Colombia	Z, S
	Nicotiana clevelandii	Cleveland's tobacco	United Kingdom	Z
	N. debneyi	Nicotiana debneyi	United Kingdom	Z
	N. glutinosa	Tobacco	United Kingdom	Z
	N.rustica	Mapacho/Markhorka	United Kingdom	Z
	N.tabacum	Cultivated tobacco	Pakistan	Z
			United Kingdom	Z
	Petunia hybrid	Petunia	United Kingdom	Z
	Physalis floridana	Physalis floridana	United Kingdom	Z
	P. peruviana	Cape gooseberry	Colombia	Z, S
	Solanum dulcamara	Bittersweet nightshade	United Kingdom*	Z
	S. nigrum	European black nightshade	Denmark	Z
			United Kingdom*	Z
			New Zealand	Z, GS
			United Kingdom	Z
			Colombia	Z

Z, zoosporangia only; S, sporosori only; GS, galls with sporosori; G, galls without sporosori.

Table 2B. 2. *Continued*

	S. physalifolium	Hairy nightshade	New Zealand	Z, GS
	S. ptycanthum	Eastern black nightshade	United States	Z, G
	S. quitoense	Naranjilla	Colombia	S
	S. sarrachoide	Hairy nightshade	United States*	Z, GS
	Sorghum bicolor	Sorghum	Pakistan	Z
Umbelliferae	*Anthriscus cerefolium*	Chervil	United Kingdom	Z
	Coriandrum sativum	Coriander	United Kingdom*	Z
Urticaceae	*Urtica dioica*	Common/Stinging nettle	United Kingdom*	Z
	Urtica urens	Annual Nettle	Denmark	Z
Violaceae	*Picea sitchensis*	Sitka Spruce	United Kingdom	Z
	Viola tricolor	Heartsease	Denmark	Z

Sources: United Kingdom (Jones & Harrison, 1969), United Kingdom * (Jones & Harrison, 1972), Denmark (Andersen *et al.*, 2002), Pakistan (Iftikhar & Ahmad, 2005), Colombia (Arcila Aristizabal *et al.*, 2013), New Zealand (Shah *et al.*, 2010), United States (Qu & Christ, 2006a), United States* (Nitzan *et al.*, 2009), and Australia (De Boer & Theodore, 1997). Z, zoosporangia only; S, sporosori only; GS, galls with sporosori; G, galls without sporosori.

2B.5. Soil-Environment Condition

The soil condition influences the survival of the pathogen, release of zoospores and the development of root infection and powdery scab. Soil environmental factors may have a direct (e.g. soil water) or indirect (e.g. soil type which affects water holding capacity) effect. This complex host-environment-pathogen interaction is illustrated in Figure 2B. 2, showing the key elements favouring disease epidemic.

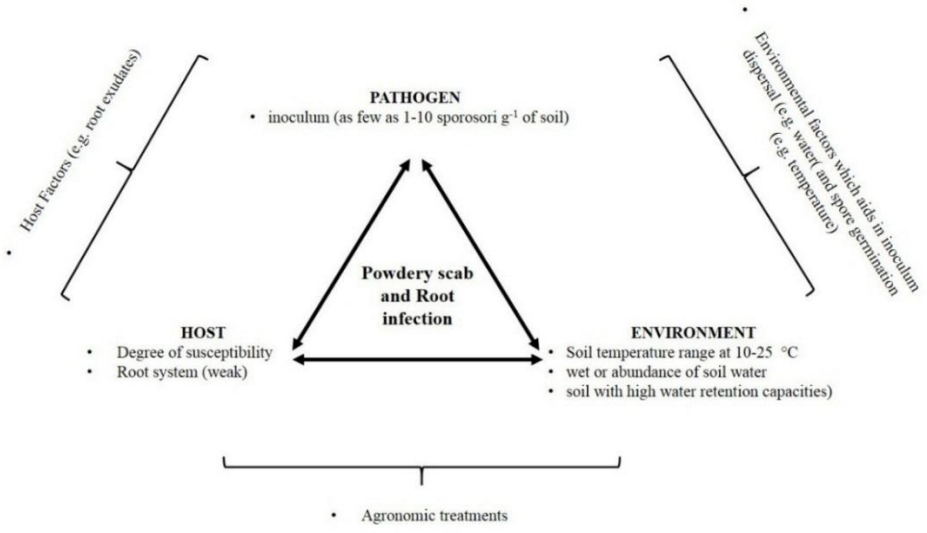

Figure 2B. 2. Important factors that contribute to powdery scab and root galling. Changes of these factors affect the amount of disease that develops.

2B.5.1. Moisture

Water, alongside inoculum and host, is a key component in disease development because zoospore release and movement to the host requires moist conditions (Kole, 1954, Merz, 2008). In a glasshouse trial, Van De Graaf *et al.* (2005) have shown that constant dampness results in greater powdery scab incidence and severity than a fluctuating moisture regime. In the field, increase in disease incidence and severity has been associated with an abundance of water through irrigation (Taylor & Flett, 1981, Kirkham, 1986, Adams *et al.*, 1987, Jellis *et al.*, 1987, Wale, 1987) and in areas which are waterlogged (Mol & Ormel, 1946, Hughes, 1980, Anonymous, 1984, Parker, 1984b). Increase in root galling was also observed in a moist-chamber experiment when soils from an infested field were constantly wet (Domfeh & Gudmestad, 2015). Decreasing the water may solve the problem, but will likely affect plant growth and yield performance due to water stress (Onder *et al.*, 2005). Dry conditions may also favour the occurrence of other destructive soil-borne diseases such as common scab, caused by *Streptomyces scabies* (Wilson *et al.*, 2001).

2B.5.2. Temperature

Soil temperature strongly influences the rate of infection and disease severity. The optimal temperature at which resting spores survive and germinate, and at which infection is severe can vary. *Spongospora subterranea* survives at temperatures below 40°C (Kole, 1954). Heating within a non-destructive range has been reported to stimulate resting spore germination in the soil. Kole (1954) conditioned resting spores by heating infested soil for 2 days at 40°C prior to planting and plants grown in this treatment showed heavier root infection than those grown in non-heated soil, presumably due to the abundance of zoospores released. Whilst it is likely that heat has stimulated zoospore release, it can be also argued that heat may have killed other microbes in the soil that are potentially inhibitory to *S. subterranea.* Nevertheless, the study indicates that the condition (e.g. temperature at which they were stored) of the inoculum prior to its interaction with the host has had a bearing in the outcome of infection. Zoospores are released into solution at a temperature between 10 to 25°C with an optimum at 15°C (Fornier *et al.*, 1996). Tuber infection favours soil temperature between 9-17°C (Van De Graaf *et al.*, 2005, Shah *et al.*, 2012) with an optimum at 12°C, whilst root infection develops well at temperatures

between 11-25°C (Kole, 1954, Van De Graaf *et al.*, 2007). The relationship of temperature and root galling has not been clearly understood due to contrasting observations in glasshouse and field experiments. Van De Graaf *et al.* (2007) reported that root galling does not form at 9°C and is severe at 17°C, but Merz *et al.* (2012) did not observe this relationship in the field.

2B.5.3. Light

Although light has not been reported to influence powdery scab, since *S. subterranea* resides in the soil, the duration and the intensity of light may affect plant condition (e.g. photosynthetic activities and vigour) and soil temperature, respectively, which may influence the outcome of infection. For instance, in other pathosystem, Foster and Walker (1947) observed that low light intensity or short day length can predispose tomato to Fusarium wilt (*F. oxysporum* f. *lycopersici*). These effects have not been investigated, and may have been overlooked, for *S. subterranea* and such may provide new knowledge on *Spongospora* disease epidemiology.

2B.5.4. pH

There are contrasting reports on the effect of pH on powdery scab in the field. Massee (1908), Reichard and Wenzl (1976), and Winter and Winiger (1983) reported that increasing pH by adding lime can reduce the severity of infection but Berkeley (1848), Melhus *et al.* (1916), Brunchorst (1887), Horne (1911), and El Fahl and Calvert (1976) observed the opposite. Studies on the release of zoospores by Diriwachter and Parbery (1991), Kole (1954), and Merz (1989) indicated that zoospores are not influenced by pH within the 5.0 to 8.0 range. If the pH does not influence zoospores, then it is likely that the addition of lime (calcium carbonate) could have affected the plant or influenced other soil chemical or physical properties which indirectly influence pathogen activity in the field. Changes of pH caused by ions (e.g. $Ca2^{+}$) could have also influence signal reception of zoospores during chemotaxis (see discussion in Chapter 2A).

2B.5.5. Physical Properties

The physical properties may directly or indirectly affect powdery scab development. Powdery scab favours soil with high water retention capacity (Prentice *et al.*, 2007), with high humus content (Wild, 1929, Sprau, 1953, Nielsen & Larsen, 2000) and with large pore spaces (Wild, 1929, van de Haar, 2000, Tuncer, 2002, Van De Graaf *et al.*, 2007). The type of soil has been found to have no effect in disease incidence in both tuber and root infection, but severity of tuber infection is much lower in clay soil (Van De Graaf *et al.*, 2005, Van De Graaf *et al.*, 2007). Clay soil also has been reported to suppress root gall formation (Van De Graaf *et al.*, 2007).

2B.5.6. Microbial Composition

No studies have yet attempted to measure the microbial activities in a soil heavily infested with *S. subterranea*. But there are indications that the relationship of *S. subterranea* to other soil-borne microbes could be symbiotic but not necessarily mutual. In previous studies, increased susceptibility of potato to other tuber diseases has been linked to powdery scab incidence (Dorojkin, 1936, Foister *et al.*, 1952, Diriwachter & Parbery, 1991). This increase in disease incidence could have been either due to *S. subterranea* providing entry for other soil pathogens or vice versa, which is a major challenge in assessing yield loss caused by root infection. There is also a possibility that yield reduction could have been caused by other, more virulent, soil microbes and not by *S. subterranea*. Commonly associated inoculum-borne microbes have not been, so far, characterised.

2B.6. Current Management Options of Powdery Scab

There is no single effective management tool for control of powdery scab. Rather, integration of two or more methods is generally employed (Falloon, 2008) and commonly include the use of less susceptible cultivars and fungicide treatments to seed tubers or soil. The following sections discusses the current control strategies categorised into measures directed towards reducing the inoculum, slowing the rate of infection and shortening the time of exposure of the host to the pathogen. Key measures include the use of certified

seed tubers, maintaining a clean/sanitised field, soil application of fungicides and the use of less susceptible cultivars.

2B.6.1. Measures that Reduce Pathogen Inocula

Several control measures before planting potato seed tubers are directed towards reducing pathogen inocula both on seed tubers and in the soil.

2B.6.1.1. Use of Certified Seed Tubers

The use of certified seed tubers is an essential practice in powdery scab management. Tegg *et al.* (2015) recently highlighted the importance of using certified seed tubers visually free of disease. They've found that planting tubers, with powdery scab lesions, greatly enhanced the incidence of root galling and powdery scab in progeny tubers. Effective detection of the pathogen on seed tubers can help limit its spread as even symptomless tubers can carry significant inoculum loads (Tegg *et al.*, 2015). For example, Norouzian *et al.* (2010) used an ELISA technique to detect contaminated mini-tubers and cull them from production. Failure to detect contaminated seed tubers can lead to the establishment of the pathogen into new regions. For example, Vakalounakis *et al.* (2013) detected the pathogen and observed powdery scab in the field where imported certified seeds (believed to have been the source of the inoculum since the pathogen has not been reported in Crete, Greece) were grown.

2B.6.1.2. Planting in Clean Field

One of the best means of avoiding powdery scab is planting potato in fields where *S. subterranea* is not present or at very low inoculum levels. Disease surveillance is an important component in managing soil inoculum precisely because one needs the knowledge of which areas/field are heavily infested and those that are relatively clean. Pathogen detection and quantitation using PCR techniques are highly recommended to determine the presence and abundance of the pathogen in the field (Brierley *et al.*, 2009, Shah *et al.*, 2014). Identifying heavily infested areas can influence the grower's choice of land use. For instance, a grower can use the clean or low inoculum field for seed tuber production.

2B.6.1.3. Crop Rotation and Alternative Hosts

Increasing the period between potato crops can reduce powdery scab, but effective rotation may require an extensive period. Sparrow *et al.* (2015) has predicted, using a non-linear model, that potato grown once every five years is still likely to remain at high risk of powdery scab. But, if crops to be rotated with potato are alternative hosts that do not allow the pathogen to complete its life cycle, they are likely to give a high reduction of soil inoculum (White, 1954). When White (1954) planted Jimsonweed (*Datura stramonium*) prior to potato, the incidence of powdery scab was reduced by as much as 30%. Infected plants grown in a field which was previously grown with *D. stramonium* had a mean severity rating of 1, whilst plants grown in control plots had a mean disease score of 4. Although White (1954) used a weed species, the risk, however, is when zoosporangia infection of the alternative hosts crop (with economic value) becomes yield-limiting and thus, knowledge of the effect of root infection in alternative host crop is very essential.

2B.6.2. Measures that Slow the Rate of Infection

Whilst reducing inoculum levels can help reduce the expression of powdery scab and root galling, it is possible that severe infection may still occur. This is because (secondary) zoospores are continuously produced during root infection and the pace of resting spore production depends on the susceptibility of the host and the soil condition. Therefore, if these factors (such as using very susceptible cultivar) favour rapid and abundant production of secondary zoospores even when the initial primary zoospore population (from resting spore) is low, severe infection or an epidemic is still likely to occur.

2B.6.2.1. Biological Control and Suppressive Soils

Research efforts on antagonistic microorganisms with potential as biocontrol agent for *S. subterranea* has been minimal. In earlier reviews (Falloon, 2008, Merz & Falloon, 2009), only one species has been reported to reduce root infection caused by *S. subterranea* (Nielsen & Larsen, 2000). To date, a few new beneficial microorganisms have shown promising results in reducing *S. subterranea* infection. A preliminary study (Hoyos Carvajal *et al.*, 2008) has revealed the presence of two *Trichoderma asperellum* strains (T-84 and T-

109) reduced the number of root galls, however, they did not examine the effect on root "zoosporangia" infection. It is very likely that gall formation may have occurred in a later period and hence, the effect on the severity of root infection in root hair and cortical cells can be overlooked. Nevertheless, avoiding or delaying the onset of galls was a significant outcome of the study. In contrast, Gilchrist *et al.* (2009) found that *T. asperellum* did not affect powdery scab. Other field experiments, using *T. harzianum,* Mycorrhizae-based commercial product (C7), *Pseudomonas fluorescens,* and *Aspergillus versicolor* (Im6-50) have also reported a reduction in the incidence and severity of powdery scab and root infection (Restrepo Duque *et al.*, 2009, Nakayama & Sayama, 2013).

2B.6.2.2. Chemical Treatment

Although chemical treatment can also be categorised as a measure that reduces pathogen inoculum, the epidemiological principle in which these chemicals operate, however, follows more on control programs based on slowing the apparent infection rate (Berger, 1977, Thangavel *et al.*, 2015). Table 2B. 3 shows some of these chemicals which are effective in reducing powdery scab. Recent field tests showed Fluazinam, mancozeb and formalin as dips (Braithwaite *et al.*, 1994, Falloon, 2008, Thangavel *et al.*, 2015) and fluazinam applied in furrow (Thangavel *et al.*, 2015) reduced root galling and powdery scab. Fluazinam is a registered pesticide for powdery scab control in New Zealand (Environmental and Protection Authority, 2015) but in Australia, fluazinam is only registered for clubroot (Australian Pesticides and Veterinary Medicines Authority, 2015). Whilst pesticides are more effective against pest and diseases in general, their continued use has been discouraged due to pesticide-associated problems (Elad *et al.*, 1992, Russell, 1995, Urban & Lebeda, 2006, Moorman, 1989, Aktar *et al.*, 2009). For example, methyl-bromide, which has long been used as a soil fumigant in the management on a wide range of soil-borne pathogens, was phased-out due to environmental issues. Some chemicals are also phytotoxic. Formaldehyde solutions, for instance, used for powdery scab control can reduce plant emergence, harvested tubers per plot, and total tuber yield of potato (Braithwaite *et al.*, 1994). Manipulating soil nutrients are a good alternative, but not as effective as the pesticides. Shah *et al.* (2014) recorded greater *S. subterranea* DNA in soil incorporated with nitrogen at 400 kg per hectare. Dipping seed tubers for 10 minutes in water at 55°C has been found to reduce powdery scab incidence but the treatment can be

detrimental to the tuber (Mackay & Shipton, 1983). Exposing tubers to steam for 10 seconds has been also found to provide good control of powdery scab (Afek & Orenstein, 2002).

Table 2B. 3. Some chemicals used to control powdery scab.

Nutrient/Chemical Name	Reference
Boron (sodium tetraborate)	Falloon et al. (2001)
Carboxin	Bhattacharyya and Raj (1986)
Dichlorophen-Na	Braithwaite et al. (1994)
Fluazinam	Falloon et al. (1996), Braithwaite et al. (1994) , Genet and Braithwaite (1992), (Thangavel et al., 2015)
Flusalfamide	Falloon et al. (1996)
Formaldehyde	Karling (1968), Braithwaite et al. (1994), Genet and Braithwaite (1992)
Mancozeb	Karling (1968), Braithwaite et al. (1994), (Thangavel et al., 2015)
Maneb	Karling (1968)
Metam sodium	Nachmias and Krikun (1988)
Methyl bromide	Nachmias and Krikun (1988)
Quintozene	Karling (1968)
Thiabendazole	Bhattacharyya and Raj (1986)
Zinc (salt)	Wale (1987)
Zinc-EDTA	Burgess et al. (1992)

2B.6.2.3. Use of Less Susceptible Cultivars

The use of resistant or less susceptible cultivars is the most desirable and a critical component in the integrated management of powdery scab. Several cultivars (Table 2B. 4) and breeding lines have been selected with resistance to powdery scab and root infection. Whilst they have shown some degree of resistance, they still succumb to infection due to high inoculum concentrations in the soil and conditions which are favourable for disease development (Merz *et al.*, 2012). Most of the commercially preferred cultivars are susceptible to root infection. Recently, Hernandez Maldonado *et al.* (2015) have demonstrated that the commercial traits of important cultivars can be potentially maintained whilst resistance to root infection is improved. They have found that plants treated with 2 and 4 mM of β-aminobutyric acid (BABA), a non-protein amino acid, have less amount of *S. subterranea* and galling in the roots.

Table 2B. 4. Some potato cultivars and their reaction to powdery scab (Merz et al. 2012).

Cultivar	Putative Resistance level
Agria	Susceptible to powdery scab
Estima	Susceptible to powdery scab
Kennebec	Susceptible to powdery scab
Desiree	Intermediate to powdery scab
Sante´	Resistant to powdery scab
Russet Burbank	Resistant to powdery scab
Gladiator	Resistant to powdery scab

Variations of resistance ratings of potato grown at various conditions and regions have been reported. In a four year cropping of ten reference potato cultivars, using a standardised scoring scale (Figure 2B. 3 and Table 2B. 5), Merz *et al.* (2012) found that different scoring methods are the main factor for the discrepancy in resistance ratings. Accordingly, the environmental conditions and soil inoculum levels play a minor role in variations of cultivar resistance reports.

There is no clear genetic link between root and tuber resistance (Merz *et al.*, 2012) but it can be argued that if cultivars have greater resistance to root infection, the infection in the tuber is likely less severe (Nakayama *et al.*, 2007) since production of (secondary) zoospores is reduced or slowed. Zoosporangia are not formed in the tuber and hence, no secondary zoospores are produced. The mechanism of resistance to powdery scab is not known, but Tegg *et al.* (2012) suggests that at least one resistance mechanism is likely similar to that of common scab of potato. They observed a positive correlation between the extent of powdery scab and common scab resistance expressed within the same clonal variants of Russet Burbank. This may likely explain why some control methods targeted specifically for common scab are also effective in controlling powdery scab (Thangavel *et al.*, 2014, Thompson *et al.*, 2014). Since no cultivar, so far, has been identified to confer resistance to both root infection and powdery scab with tuber qualities which are more preferred by the consumer and the processing industry, the use of existing less-susceptible cultivars is recommended to mitigate the effects of high soil inoculum pressure and conditions favouring disease development, combined with the other measures mentioned earlier.

Table 2B. 5. Standardised root galling severity scores (http://www.spongospora.ethz. ch/LaFretaz/scoringtablegalls.htm).

Score	Symptoms observed
0	no galls
1	1-2 galls
2	3-10 galls, mostly small (< 2 mm in diameter)
3	>10 galls, most in clusters
4	many galls, regularly distributed

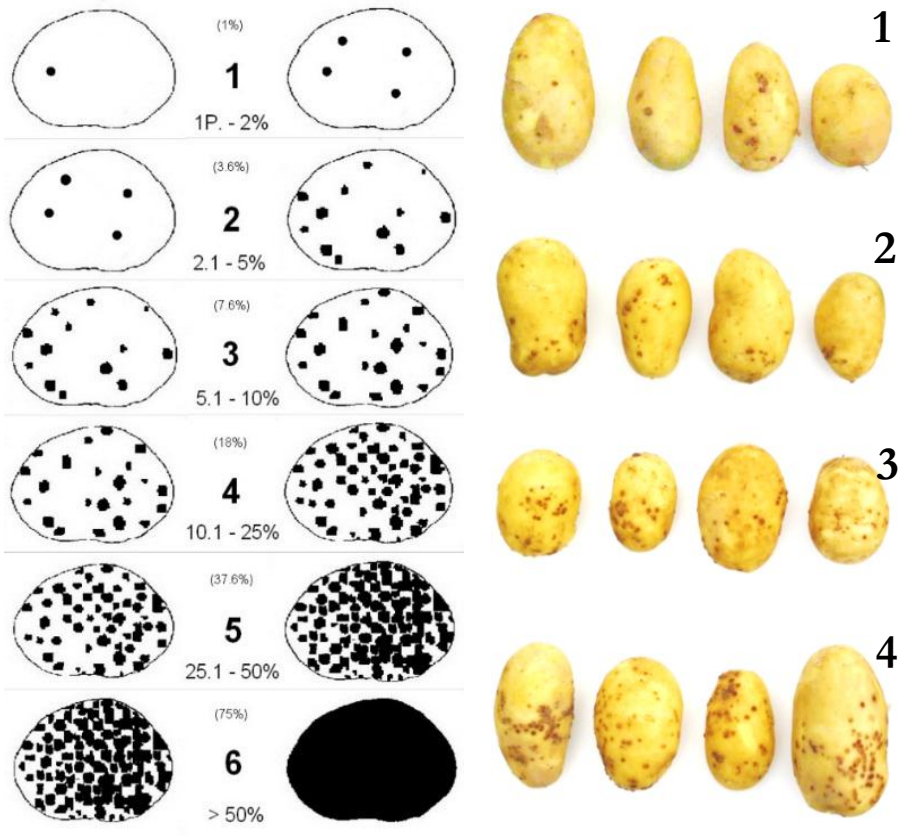

Figure 2B. 3. Graphical representation of the standardised powdery scab severity scores. Diseased potato tuber (cv. Agria) were obtained from http://www.spongospora.ethz. ch/LaFretaz/scoringtable.htm, and used with written permission from Ueli Merz (ETH Zurich, Switzerland).

2B.6.3. Measures that Shorten the Time of Exposure

Thangavel *et al.* (2015) examined the effect of delayed inoculation of *S. subterranea* on root infection. They found that the longer the time the host is exposed to the pathogen, the more severe the root infection and the higher *S. subterranea* DNA in the roots. Therefore, if the disease develops at the stage where the plant is close to maturity there will be less risk of an epidemic. Berger (1977) suggested two ways to shorten the time of exposure of the pathogen to the host, either use cultivars with well-developed root system (vigorous roots) or use short season cultivars whilst maintaining adequate soil fertility to enhance crop growth. Accordingly, at the start of infection, the pathogen would have less time before it reaches the level of economic loss. Therefore, developing potato cultivars with more robust root system is likely another aspect to be integrated in the potato breeding program.

2B.7. Research Prospects and Conclusion

Dormancy is an important aspect of pathogen survival in the soil, but it is unknown whether *S. subterranea* resting spores enter the dormant state. Alongside investigating specific soil-environmental factors the pathogen actively responds to (Chapter 2A), investigating resting spore dormancy is another key aspect to examine (Chapter 3). Such knowledge will be useful in disease risk assessment and management.

There is an apparent lack of knowledge on the value of actual losses caused by powdery scab. Although powdery scab has been widely acknowledged as an impediment in potato production, determining the actual scale of the disease's impact will most likely assist key industry and government decision-makers in providing more support to growers (particularly the access to affordable seed DNA testing), the potato industry and research institutions, and in elevating research programs aiming towards developing durable control measures. Another arising issue is the effect of root infection on yield. It is not yet clear if root infection reduces tuber yield. Falloon *et al.* (2016) observed reduction of tuber yield, but Johnson and Cummings (2015) reported otherwise. Further evaluation would be needed. An aspect to improve is the method of inoculum preparation, in which the aim is to purify the inoculum and eliminate other potential inoculum-borne microbes which may exacerbate the yield effect. Bulman *et al.* (2011) have shown that root galls can be surface-sterilised and maintained on a culture medium (without

contamination), which indicates that the sporosori-inoculum (in the galls) are relatively clean and can be used to infect plants *in vitro*. Infected plants can be transplanted to glasshouse or in the field for assessment.

An understanding of how beneficial microbes contribute to disease suppression is still lacking, but progress on the identity of candidate microbes for biological control has been one of the milestones in powdery scab research in the last 7 years. But more research still needs to be done both in controlled and field conditions to elucidate the control mechanism and the interaction of these potential bio-control agents with the plant and to other soil microorganisms. For instance, the microorganisms reported (see section earlier) have been shown to reduce the infection and severity, but it remains unclear if these microorganisms directly (e.g. inhibition of resting spore germination or competing with zoospores) or indirectly (e.g. improved plant root architecture and growth, altered soil chemistry, or promoted microbial activities of other soil-borne microorganisms) affected *S. subterranea*. The same approach applies to organic soil-amendments (presumed to have high microbial population) if found to have antagonistic effects on *S. subterranea*. Additionally, utilising disease suppressive soils can be also explored for *S. subterranea*. However, knowledge on the function and characterisation of the biological, physical and chemical properties of disease suppressive soils using appropriate methods is required to understand the nature of its suppressiveness to be able to use them effectively.

There is an increasing interest for safe and sustainable approach in the control of pests and diseases of major crops. This is largely driven by controversies about the use of pesticide-based chemicals which are labelled costly, unsafe to user's health and toxic to the environment. This trend would likely to be seen in powdery scab chemical management and therefore, effective approaches alternative to pesticide-based chemicals are warranted. Several studies in other plant-pathogen pathosystems have documented that chemicals in the root exudate, which interact with the pathogen, can be used for disease management as an alternative to pesticides. There is, however, a lack of knowledge of the compounds released by potato and other host species roots during exudation. Therefore, there is a need to first characterise the root exudate chemical properties (Chapter 4 and Chapter 5), and root exudates that are biologically active to *S. subterranea* (see Chapter 2A for further discussion).

There are many alternative hosts reported, but it is uncertain if they can all be used for disease control (as rotation crop or bait plant). If they are, understanding the potential effect of root infection of these plants, particularly those with economic value is essential. For instance, planting common poppy prior to potato may help reduce the inoculum, but *S. subterranea* may affect the yield or the alternative host-plant's important commercial qualities.

Whilst most reviews and studies on powdery scab already acknowledged the importance of using resistant potato cultivars as the most durable and effective control, studies understanding the mechanism of resistance and characterising resistance (*R*) genes have just recently begun. Sources of resistance to *S. subterranea* root galling are available (Nitzan *et al.*, 2008, Nitzan *et al.*, 2010) and these breeding lines will be useful materials for future potato breeding programs. Improvement of root growth and architecture can be also included in potato breeding programs. Additionally, knowledge of the genes associated with resistance will be useful in developing biomarkers to expedite the screening of resistant lines. However, in developing new lines with resistance to powdery scab and root infection, the commercial merits (e.g. tuber cooking quality, acceptable to French fry standards) need to be considered and therefore, other methods with high retention efficiency of important tuber commercial traits should be explored. For instance, Wilson *et al.* (2010b) developed a common scab resistant line of Russet Burbank (one of the world's most important potato cultivars), with essential tuber qualities indistinguishable from that of the parent cultivar (Wilson *et al.*, 2010a). The potato lines were developed using a cell selection technique that utilises a toxin from the pathogen, as selective agent, killing potato cells that are likely susceptible to the disease. Certain chemicals have been also reported to induce resistance without significantly altering plant characteristics. Hernandez Maldonado *et al.* (2012) have shown that susceptible cultivars receiving BABA treatments had a less severe infection than those that did not receive the chemical treatment.

The knowledge of controlling powdery scab relies on the depth of understanding of its epidemiology. Knowledge of the powdery scab epidemiology is paramount in deciding what management strategies should be used under certain condition. Since there is yet no effective, durable control, the understanding of the epidemiological aspects in powdery scab pathosystem is far from complete but, nevertheless, has improved. The challenge now is to fill in the missing links and knowledge gaps to further improve the

understanding of disease epidemiology and develop control measures through continued collaborative efforts among stakeholders – growers, the potato industry, researchers and other relevant institutions – of whom will mainly benefit when a durable and cost-effective control of powdery scab is achieved. Finally, increasing the availability of potato by avoiding losses due to *Spongospora* disease will contribute to the global aim of securing potato as one of the staple crops of the increasing human population.

Chapter 3. Resting spore dormancy and infectivity characteristics of the potato powdery scab pathogen *Spongospora subterranea*

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3.1. Abstract

The soil-borne potato pathogen *Spongospora subterranea* persists in soil as sporosori, which are aggregates of resting spores. Resting spores may germinate in presence of plant or environmental stimuli, but direct evidence for resting spore dormancy is limited. A soil-less tomato bait-plant bioassay and microscopic examination were used to examine features of *S. subterranea* resting spore dormancy and infectivity. Dried sporosori inocula prepared from tuber lesions and root galls were infective after both short and long term storage (1 week to 5 years for tuber lesions and 1 week to 1 year for root galls) with both young and mature root galls inocula showing infectivity. This demonstrated that a proportion of all *S. subterranea* resting spores regardless of maturity exhibit characteristics of stimuli-responsive dormancy, germinating under the stimulatory conditions of the bait-host plant bioassay. However, evidence for constitutive dormancy within the resting spore population was also provided as incubation of sporosorus inoculum in a germination-stimulating environment did not fully exhaust germination potential even after 2.4 years. We conclude that *S. subterranea* sporosori contain both exogenous (stimuli-responsive) and constitutively dormant resting spores, which enables successful host infection by germination in response to plant stimuli and long term persistence in the soil.

Keywords: Spongospora root disease, potato pathogen, plasmodiophorid, inoculum persistence, tomato bait-plants

3.2. Introduction

The soil-borne pathogen *Spongospora subterranea* (Wallr.) Lagerh. causes the tuber disease powdery scab and root diseases of potato (Falloon *et al.*, 2016). *Spongospora* diseases are one of the most economically significant problems in potato production worldwide (Chapter 2A, Wilson, 2016). Presence of powdery scab lesions downgrades fresh market tuber value (Harrison *et al.*, 1997), and can result in failure of seed tuber certification (Tegg *et al.*, 2014). Root infection can impact plant growth and yield (Falloon *et al.*, 2016). Current control practices for *Spongospora* diseases in potato are unsatisfactory. Some studies show success of pesticides in delaying disease development or reducing disease impact (Falloon, 2008, Thangavel *et al.*, 2015), but the mechanisms of disease suppression are not fully understood.

Dormancy is an important characteristic of many spore forming plant pathogens that enables persistence in the soil in the absence of their hosts (Cochrane, 1974, Deacon & Deacon, 2005, Feofilova *et al.*, 2011). Dormant spores may be sensitive to exogenous factors (e.g. moisture, temperature or phytochemicals) which trigger spore activation or germination (stimuli responsive, exogenous or environmental dormancy). The presence of stimuli probably indicates that an unfavourable condition has ceased and/or presence of a suitable host (Cochrane, 1974, Deacon & Deacon, 2005, Feofilova *et al.*, 2011). Exogenous dormancy may also require sources of energy for activation (Cochrane, 1974, Schroth & Hildebrand, 1964). Constitutive or endogenous spore dormancy also occurs, in which spores later respond to exogenous stimuli when such dormancy ceases. The germination of spores from this type of dormancy is delayed by the innate properties of the dormant stage such as barriers to the penetration of nutrients, metabolic blocks or self-inhibitors (Sussman, 1965, Cochrane, 1974). Dormancy may also cease when the integrity of the spore walls is weakened (Sussman, 1965, Cochrane, 1974, Deacon & Deacon, 2005, Feofilova *et al.*, 2011).

Spongospora subterranea is a plasmodiophorid (Braselton, 1995). The plasmodiophorid plant pathogens (*S. subterranea, Plasmodiophora brassicae, Polymyxa* spp.) survive in the soil as resting spores and infect their hosts as motile zoospores. Resting spores have thick cell walls that protect the zoospores during unfavourable conditions (Lahert & Kavanagh, 1985). *Spongospora subterranea* resting spores, and those of most other plasmodiophorids, are aggregated in sporosori, whilst *P. brassicae* has individual resting spores. The *S. subterranea* sporosorus is complex in which internal resting spores are

protected by outer layers of the sporosorus (Falloon *et al.*, 2011). Morphological assessment of *S. subterranea* sporosori indicated only a proportion of resting spores were released as zoospores over the period of assessment (Falloon *et al.*, 2011). The authors suggested this allowed the pathogen to maintain inoculum potential over an extended period. Persistence of *S. subterranea* within the soil has been linked to resting spore dormancy (Harrison *et al.*, 1997, Merz, 2008, Falloon, 2008, Chapter 2A), but dormancy has not been widely investigated, with few empirical observations reported to date. Different reports indicated that resting spores can survive for many years, although most were from observations on the re-occurrences of infection in the field (Harrison *et al.*, 1997) with few reports based on direct resting spore examination (Kole, 1954, Merz, 1989, De Boer, 2000). Persistence of resting spores may also vary with incubation conditions. Merz (1989) suggested infectivity of resting spores in moist soil decreased with increasing time of storage.

The present study aimed to determine if *S. subterranea* resting spores exhibit characteristics of exogenous and constitutive dormancy. Greater understanding of the survival and germination of *S. subterranea,* could provide valuable knowledge for formulating and implementing *S. subterranea* disease management strategies.

3.3. Materials and Methods

3.3.1. Source and Preparation of Sporosori-inoculum

Seven *S. subterranea* sporosori samples were collected from infected potato plants from different fields in NW Tasmania. The samples included four sourced from powdery scab-affected tubers and three from mature potato root galls (dark brown to black coloured) which were processed as described below and stored for periods of 1 week to 5 years (tuber inocula) or 1 week to 1 year (gall inocula; Table 3. 1). Young, cauliflower-like, white to cream-white coloured galls were also sampled and stored for 1 week after processing (Figure 3. 1; Table 3. 1).

Sporosori were sampled and processed following the method of Merz (1989), with modifications. For each tuber sporosori sample, all powdery scab lesions (10-50 per tuber) were excised from *c.* 20 diseased tubers, collected from the same field, using a vegetable peeler. For the gall sporosori samples, all young or mature galls from *c.* 20 plants (50 – 60 galls per sample), collected from the same field, were cut from diseased potato roots and

washed with running tap water. All sporosori samples were oven-dried for 4 days at 40 °C, powdered using a mortar and pestle and stored at 4 °C, in the dark, until testing. Tuber tissues free from visible lesions were similarly processed as controls.

3.3.2. Tomato Bait-Plant Bioassay

The infectivity of each sporosori sample was assessed using a simplified soil-less bioassay method (Merz, 1989). Tomato were used as bait-plants because they are very susceptible to *S. subterranea* root zoosporangium infection (Merz, 1989), allowing rapid assessment of infection. Tomato (*Lycopersicon esculentum* cv. Grape) seeds were sown in nursery grade potting mix soil (1:1:8 mix of peat, course sand, and composted pine bark) in a glasshouse maintained at 20±2 °C. Two weeks after sowing, seedlings were each gently uprooted, roots washed with running tap water to remove adhering soil, and placed in a McCartney bottle containing 20 mL of nutrient solution (Shah *et al.*, 2012) for 24 hours. Test plants were then individually placed in a McCartney bottle containing 20 mL of deionised distilled water (DW, 0.059 μS/cm conductivity) to which 1 mg of sporosorus inoculum was added. Plants were incubated at 15-18 °C, 60% humidity with DW added as necessary to maintain a 20 mL volume in each McCartney bottle. After 4 weeks, plants were removed from the solution, roots were excised and washed with running tap water. Separate root samples were selected from the top, mid-section and bottom of each root profile, mounted on microscopic slides and stained with 0.1 % Trypan blue in lactophenol for 15-30 min. The intensity of zoosporangium infection was assessed using the scale 0 – no infection, 1 – sporadic, 2 – slight, 3 – moderate, 4 – heavy, 5 – very heavy (Merz *et al.*, 2004) (Figure 3. 2). Root infection scoring and confirmation of zoosporangia were assessed under a light microscope (DM 2500 LED, Leica Microsystem, Germany) at 200x and 400x magnification, respectively (Hernandez Maldonado *et al.*, 2012). Three to eight plants per treatment were tested with the three root samples per plant (top, middle and bottom) examined and recorded separately.

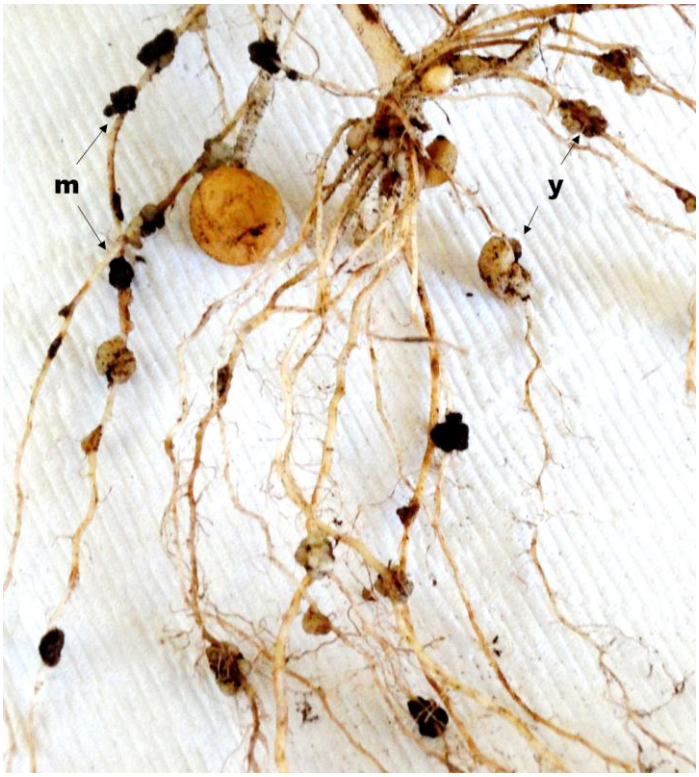

Figure 3. 1. White-cream coloured**,** young (y) and dark brown to black mature (m) galls in potato roots.

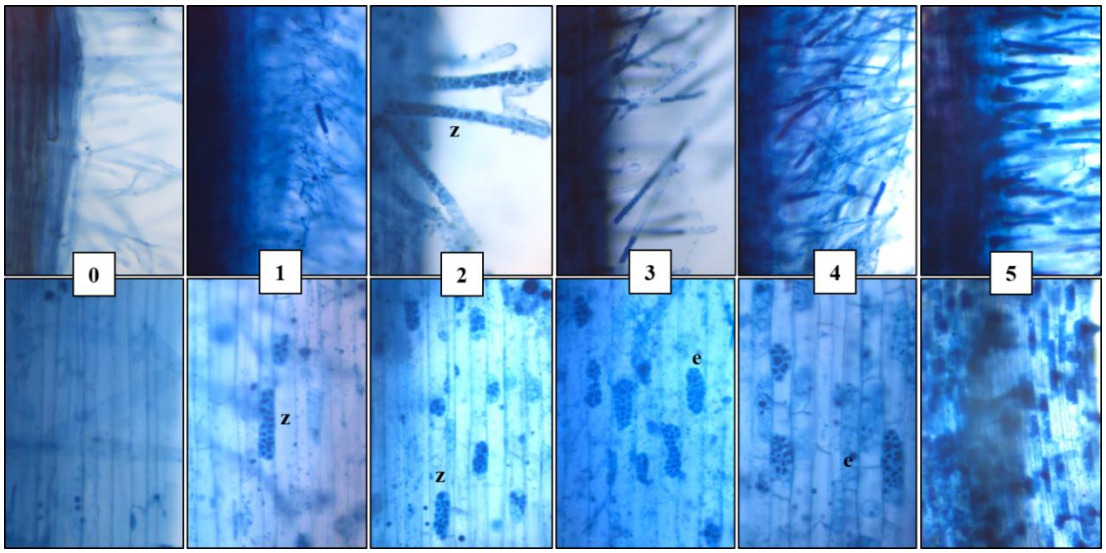

Figure 3. 2. Representative photomicrographs of the intensity of zoosporangium infection by *Spongospora subterranea* in tomato (cv. Grape) root hairs (upper section; observed at 200x magnification) and root surface epidermal cells (lower section; observed at 400x magnification). Root infection was microscopically examined, and severity was evaluated using a 0-5 scale (Merz *et al.*, 2004).

3.3.3. Viability and Infectivity of Dry-stored Sporosori-inoculum

To assess sporosori persistence in dry storage and capacity for host stimuli-induced germination, dried and powdered sporosorus inoculum from tuber lesions were stored for 1 week, 1, 3 or 5 years at 4 °C in the dark before testing for infectivity using eight tomato bait-plants per inoculum source using the method described above. Dried and powdered sporosorus inoculum from root galls incubated for 1 week (young and mature galls), 9 months or 1 year were similarly tested for infectivity using five tomato bait-plants per inoculum source. Tomato plants (three per treatment) incubated in inoculum-free DW and in DW with powdered potato tuber skin free from powdery scab lesions were included as negative experimental controls. A total of 24 and 15 root samples were assessed for each tuber and root gall inoculum treatment respectively.

3.3.4. Viability and Infectivity of Sporosori-inoculum stored in a Germination Stimulatory Environment

To confirm the germination stimulatory effect of nutrient solution (modified Hoagland's solution) (Falloon *et al.*, 2003) on resting spore germination (Merz, 1989; Merz, 1997) 1 mg of dried inoculum from tuber lesion material that had been stored for 1 year was added to 2 mL of nutrient solution and to 20 mL DW and incubated at 15-18 °C in the dark. Three aliquots (35 µL each) were sampled daily and examined microscopically for presence of *S. subterranea* zoospores distinguished by morphology (size and biflagellate form; Kole, 1954; Merz, 1997) and characteristic swimming patterns (Merz, 1992).

To test longevity of infectivity under stimulatory conditions and presence of constitutive dormancy of resting spores, dried sporosorus inoculum from tuber lesion material stored for 6 months (20 mg) was added to 500 mL of nutrient solution and incubated at 18-20 °C, in the dark for up to 29 months (2.4 years). Subsamples (1 mL) were taken at 2 weeks, 2 months and 2.4 years' incubation time and placed in McCartney bottles each containing 19 mL DW and the inocula was assessed for infectivity using the tomato bait-plant assay described above.

3.3.5. Data Analysis

Data and statistical analyses were performed using SPSS® statistical software (Version 22, Armonk NY). Analysis of variance was performed to determine the variation between three or more treatments. The Fisher's least significant difference (LSD) test, at 0.05 level of probability, was used for multiple comparison of means. The means of zoosporangium severity scores from tuber and gall resting spores, within the same age, were compared using an independent t-test analysis. The association between zoosporangium severity and age of inoculum was determined using the Pearson's *r* correlation analysis.

3.4. Results

3.4.1. Viability and Infectivity of Dry-stored Sporosori Inoculum

All dried sporosorus inocula, after the incubation period, were able to efficiently infect tomato seedlings once activated by suspending in DW with the host plant seedlings (Table 3. 1; Figure 3. 2). For each assay 18-24 root segments from the eight bait plants (tuber inocula) or 12-15 root segments of the five bait plants had zoosporangial infection. The control treatments (nine root segments from 3 plants treated with healthy powdery tuber tissue and DW) did not show any zoosporangia. Whilst infection severity levels are indicative only, resultant mean disease severity statistically varied between tuber inocula ($P<0.001$) and between gall inocula ($P<0.001$) with dried tuber inocula samples stored for 1 week or 3 years showing greater disease severity than those stored for 1 year or 5 years, and root gall inocula stored for 9 months and 1 year having lower disease severity than those stored for 1 week. The age of tuber or root gall inocula did not correlate with severity of zoosporangium root infection ($r=-0.968$, $P=0.929$: tuber; and $r=-0.071$, $P=0.052$: root gall). There were no differences found in infection success (15 root segments from five plants each) nor disease severity between young (mean score = 3.6) and mature (3.4) gall inocula stored for 1 week.

3.4.2. Viability and Infectivity of Sporosori Inoculum Stored in a Germination Stimulatory Environment

Adding dried sporosori inoculum to nutrient solution stimulated earlier and greater release of zoospores than the DW control (Table 3. 2). Dried inoculum incubated

in nutrient solution remained infective for at least 2.4 years when subsamples were tested with the tomato bait plant assay (Table 3. 3). The length of incubation did not affect the number of infected root segments observed (all nine segments from three bait-plants showing infection for each treatment) nor mean disease severity (3.3 – 3.7).

3.5. Discussion

Resting spore dormancy is important for *S. subterranea* longevity in absence of suitable host plants (Harrison *et al.*, 1997), but there have been no empirical studies on resting spore dormancy. It is well known that *Spongospora subterranea* resting spores in sporosori can persist in the soil for many years. Longevity of resting spore viability and subsequent capacity to infect host plants has been demonstrated. De Boer (2000) found host plants introduced to 4-year-old resting spore-infested soils succumbed to infection. Resting spores persisting in dry soils for 6 years also remained capable of causing infection when host plants were introduced (Kole, 1954). Furthermore, several authors have suggested that resting spores can survive for more than 10 years in the soil following observations from field sites with extended periods between potato crops (Merz, 2008, Falloon, 2008, Sparrow *et al.*, 2015). However, it remains unclear whether resting spore dormancy was due to constitutive or exogenous mechanisms. Measurement of pathogen presence and abundance in soil and interpretation of these data can be problematic. Polymerase chain reaction and enzyme-linked immunosorbent assay methods can detect and enumerate pathogen levels in the soil (Brierley *et al.*, 2009), but these methods do not necessarily indicate pathogen viability nor differentiate between dormant and infective inocula. Certain chemical stains can detect viability of spores (Riss *et al.*, 2013), but while stains had been used to analyse *P. brassicae* resting spores (Takahashi, 1994), the complex sporosorus structures of *S. subterranea* negates the usefulness of this approach. This has probably added to difficulties in obtaining direct empirical evidence linking dormancy to *S. subterranea* resting spore persistence. The use of host bait-plants allows the direct observation of infections and provides evidence of the presence of infective pathogen inoculum (Merz, 1989). Whilst only modest numbers of test plants were used in these trials, and thus the infection rates presented are approximate only, they do clearly indicate efficient infectivity occurred.

Table 3.1. Incidence and mean severity (0-5) of *Spongospora subterranea* zoosporangium root infection in tomato bait plant roots, from resting spores from different sources and different ages.

Resting spore source and age [1]		Collection	Disease incidence [2]			Disease Severity (0-5) [3]			Total No. of Roots Assessed	Mean disease severity [3]
			Top	Middle	Bottom	Top	Middle	Bottom		
Tuber lesions										
1-week old		This study	8/8	8/8	8/8	3.9 ± 0.3	4.3 ± 0.4	4.6 ± 0.3	24	4.3 ± 0.2 c*
1-year old		This study	6/8	6/8	6/8	1.4 ± 0.4	1.3 ± 0.4	1.1 ± 0.3	24	1.3 ± 0.2 a
3-year old		TIA Collection	8/8	8/8	8/8	4.0 ± 0.3	4.3 ± 0.3	4.5 ± 0.3	24	4.3 ± 0.1 c
5-year old		TIA Collection	8/8	8/8	8/8	2.5 ± 0.5	2.6 ± 0.5	2.8 ± 0.4	24	2.6 ± 0.2 b
P value										*<0.001*
Root galls										
1-week old	Young	This study	5/5	5/5	5/5	3.8 ± 0.4	3.6 ± 0.5	3.4 ± 0.4	15	3.6 ± 0.2 b
1-week old	Mature	This study	5/5	5/5	5/5	3.4 ± 0.2	3.4 ± 0.4	3.4 ± 0.5	15	3.4 ± 0.2 b
9-month old	Mature	This study	4/5	4/5	4/5	1.8 ± 0.6	1.6 ± 0.6	1.4 ± 0.4	15	1.6 ± 0.3 a
1-year old	Mature	This study	4/5	4/5	4/5	1.6 ± 0.5	1.6 ± 0.5	1.6 ± 0.4	15	1.6 ± 0.3 a
P value										*<0.001*
Controls (no *S. subterranea*)										
Dried and powdered healthy tuber skin			0/3	0/3	0/3	0	0	0	9	0.0
Water			0/3	0/3	0/3	0	0	0	9	0.0

[1] Age of resting spores from the day of processing until testing. All inocula were dried at 40 ºC for 4 days and ground to a powder. Young root galls were white to cream-white and mature root galls were dark brown to black.

[2] Number of roots sample infected/total number of roots assessed.

[3] Root infection was evaluated using the scale 0 – no infection, 1 – sporadic, 2 – slight, 3 – moderate, 4 – heavy, 5 – very heavy (Merz *et al.*, 2004).

[*] Means followed by different letters within each resting spore type were significantly different at P=0.01, using Fisher's LSD test. Values are means ± standard error.

Table 3. 2. Mean numbers of *Spongospora subterranea* zoospores counted in 35 µL aliquots following incubation of 1 mg sporosori inoculum in 2 mL of nutrient solution or distilled water.

Treatment	Days incubation						Cumulative count
	4	7	10	13	17	20	
Nutrient Solution	0	9.7 ± 2.3	134.7 ± 6.8	65.3 ± 7.6	18 ± 2.4	18 ± 7.8	245.7
Distilled Water	0	0 ± 0.0	0	0	12.3 ± 2.6	58.7 ± 2.0	71
P value	-	*0.014**	*0.000**	*0.001**	*0.188*	*0.007**	

Inoculum was from tuber lesions dried at 40 ºC for 4 days, ground to a powder, and stored for 6 months prior to use. Inocula were incubated in treatment solution at 18-20 °C.

* Means zoospore population between the solutions were significantly different at $P<0.01$, using an independent t-test analysis. Values are means ± standard error.

Table 3. 3. Incidence and mean severity (0-5) of *Spongospora subterranea* zoosporangium root infection in tomato bait plant roots, from sporosori inoculum held in nutrient solution for varying periods.

Period of incubation [1]	Disease incidence [2]			Disease Severity (0-5) [3]			Total No. of Roots Assessed	Mean disease severity [3]
	Top	Middle	Bottom	Top	Middle	Bottom		
2 weeks	3/3	3/3	3/3	3.3 ± 0.35	3.3 ± 0.41	3.3 ± 0.26	9	3.3 ± 0.20
2 months	3/3	3/3	3/3	3.7 ± 0.37	3.7 ± 0.41	3.7 ± 0.35	9	3.7 ± 0.21
2.4 years	3/3	3/3	3/3	3.7 ± 0.26	3.3 ± 0.25	3.0 ± 0.27	9	3.3 ± 0.15
P value								n.s. (0.383)

Inoculum was from tuber lesions dried at 40 ºC for 4 days, ground to a powder, and stored for 6 months prior to use.

[1] Length of time resting spores were incubated in nutrient solution at 18-20 °C.

[2] Number of plants infected over total number of plants assessed.

[3] Root infection was evaluated using the scale 0 – no infection, 1 – sporadic, 2 – slight, 3 – moderate, 4 – heavy, 5 – very heavy (Merz *et al.*, 2004). n.s. = not significant at 0.05 level of significance by Fisher's LSD test. Values are means ± standard error.

Our study clearly shows that sporosori inocula harvested from both tubers and root galls that were dried prior to storage were infective across a wide range of storage periods from 1 week to 1 year (root gall) or 5 years (tuber) and that both young (white-cream) and mature (brown-black) root galls stored for 1 week contain infective zoospores. This demonstrated that tomato bait-plants were capable of stimulating zoospore release from both freshly produced and aged inocula providing evidence for stimuli-responsive dormancy of *S. subterranea* resting spores. Observations of the stimulation of *S. subterranea* resting spore germination has been previously suggested as providing evidence for exogenous dormancy processes. Merz (1993) first reported that host plant root exudates can stimulate or induce *S. subterranea* resting spore germination using root infection as an indicator. Fornier *et al.* (1996) demonstrated direct effects of root exudates to stimulate resting spore germination, quantifying zoospores in an exudate solution. We add to the current knowledge by demonstrating that both recently formed and aged *S. subterranea* resting spores may be stimulated to germinate by the presence of a stimulatory host and that there is no requirement for extended ageing for at least some *S. subterranea* resting spores germinate in presence of a host. We also confirm that defined nutrient solution provides a germination stimulus for *S. subterranea* resting spores (Merz, 1989, Merz, 1997) resulting in more rapid and greater numbers of zoospores released than water alone. These data provide direct evidence for exogenous dormancy characteristics of *S. subterranea* resting spores.

We also show sporosori inocula maintained under stimulatory conditions (defined nutrient solution) for extended periods of time remain capable of releasing infective zoospores in the presence of tomato bait-plants. This suggests a proportion of the resting spores within the sporosori inocula are not stimuli-responsive otherwise we would expect germination to be fully exhausted within a relatively short period of exposure to such germination conducive conditions. Instead a proportion of the resting spores show characteristics of constitutive dormancy that become responsive to stimuli after constitutive dormancy factors have been removed over time. Indirect evidence for presence of constitutive dormancy within populations of *S. subterranea* resting spores was previously provided by Falloon *et al.* (2011). Through observation of sporosori over 5 to 8.5 hours they found the majority of resting spores within a sporosorus did not release zoospores, and were either non-viable or remained dormant.

Features of *S. subterranea* resting spore dormancy observed in this study were similar to those reported for *P. brassicae* (Ohi *et al.*, 2003, Friberg *et al.*, 2005). *Spongospora subterranea* and *P. brassicae* are both plasmodiophorids and their resting spores have morphological similarities (Braselton, 1995, Braselton, 2001). *Plasmodiophora brassicae* persistence in the soil has also been linked to resting spore dormancy (Macfarlane, 1970, Kageyama & Asano, 2009) and their germination may be stimulated by nutrient solution (Friberg *et al.*, 2005) and host root exudates (Ohi *et al.*, 2003).

The findings from the present study strengthen understanding of the nature of survival and germination of *S. subterranea* resting spores. This knowledge could be valuable for disease management. In absence of the host, germination stimulants could be used to prematurely trigger release of the relatively short-lived zoospores (Karling, 1968), leading to a reduction of the soil inoculum potential (Donald & Porter, 2014). An example of the use of this approach is shown by the addition of derivatives from garlic or onion containing diallyl disulfide to soil infested with *Sclerotium cepivorum*, the fungus which causes onion white rot. These compounds stimulated sclerotial germination in absence of *Allium* hosts and reduced soil inoculum levels and resultant disease severity in subsequent onion crops (Davis *et al.*, 2007). A similar approach using specific compounds from brassica root exudates to stimulate germination of *P. brassicae* resting spores in absence of host plants has recently been proposed (Mattey & Dixon, 2015).

Our study also highlights the limitations of such an approach. As a proportion of *S. subterranea* resting spores exhibit constitutive (non-stimuli responsive) dormancy, inoculum reduction may only be partial. Similarly, certain constitutively dormant sclerotia of *Sclerotium cepivorum* failed to respond to diallyl disulfide stimuli (Coley-Smith *et al.*, 1987). However, if *S. subterranea* soil inoculum could be reduced to a level that will reduce or delay infection (Thangavel *et al.*, 2015), then the treatments may be highly beneficial. A detailed investigation of the form and concentration of stimulant and longevity of exposure of resting spores to germination stimulants is warranted.

In conclusion, this study confirms *S. subterranea* resting spores from tuber lesions and root galls may survive dried for an extended period of time without affecting resting spore infectivity. But evidence suggest that resting spores exhibit characteristics of a constitutive (innate) dormancy and thus, whilst it may be possible to utilise exogenous germination stimuli to encourage germination of resting spores in the absence of host plants to deplete soil inoculum and reduce disease (Davis *et al.*, 2007, Falloon, 2008), we

would expect constitutively dormant resting spores to remain as infective sources of inoculum.

Chapter 4. Metabolomes of potato root exudates: compounds that stimulate resting spore germination of the soil-borne pathogen *Spongospora subterranea*

4.1. Abstract

Root exudation has importance in soil chemical ecology influencing rhizosphere microbiota. Prior studies reported root exudates from host and non-host plants stimulated resting spore germination of *Spongospora subterranea,* the powdery scab pathogen of potato, but the identity of stimulatory compounds was unknown. This study showed that potato root exudates stimulated *S. subterranea* resting spore germination, releasing more zoospores at an earlier time than the control. We detected 24 low-molecular weight organic compounds within potato root exudates and identified specific amino acids, sugars, organic acids and other compounds that were stimulatory to *S. subterranea* resting spore germination. Given several stimulatory compounds are commonly found in exudates of diverse plant species we support observations of non-host specific stimulation. We provide knowledge of *S. subterranea* resting spore biology and chemical ecology that may be useful in formulating new disease management strategies.

Keywords: Powdery scab, root infection, plasmodiophorid, HILIC UPLC-MS, zoospore release, metabolomics

4.2. Introduction

Spongospora subterranea (Wallr.) Lagerh. causes powdery scab and root disease in potato (Falloon *et al.*, 2016). *Spongospora* diseases are major problems in potato production worldwide. The pathogen downgrades the tuber fresh market-value, causes substantial economic losses in the potato processing industry (Wilson, 2016), and leads to failure of seed tuber certification and subsequent losses in product value for seed potato producers

(Tegg *et al.*, 2014). Primary zoospores initiate infection after release from germinating resting spores that are formed into aggregates known as sporosori (Kole, 1954). Zoospores migrate to host roots, where they encyst and establish infections (Keskin & Fuchs, 1969) which develop into plasmodia and may mature as zoosporangia or resting spores (Braselton, 1992). Zoosporangia contain secondary zoospores which are sources of secondary infection in tubers and roots after release into the soil environment. When resting spores are released back into the soil they become the new generation of primary inoculum. As such, the germination of *S. subterranea* resting spores is the first crucial step in disease development. Despite its importance, knowledge of the processes and the factors influencing resting spore germination is limited (Chapter 2A). Root exudates play a critical role as stimulants of *S. subterranea* resting spore germination (Kole, 1954, Merz, 1993, Fornier *et al.*, 1996). Prior studies report a lack of host specificity with exudates from both hosts and non-hosts capable of stimulating germination. However, no attempts have been made to identify any stimulatory compound(s) present in the root exudates.

An estimated 40-50% of the carbon fixed by plants is released as root exudates, mostly as low molecular weight organic (LMWO) compounds, making root exudation a significant carbon cost to the plant (Bais *et al.*, 2006, Whipps, 1990) Root exudates play important roles in rhizosphere chemical ecology influencing interactions between the plant and other soil biota. They may function as, but are not limited to, chemoattractants, inhibitors or stimulants of microbial growth (Bais *et al.*, 2006) and may influence the colonization and activation of root-infecting pathogens (Schroth & Hildebrand, 1964, Nelson, 1990) The response of soil microbes may vary depending on the root exudate's organic composition. For instance, peanut root exudates differing in LMWO compound composition have varying influence on spore germination of soil-borne peanut pathogens (Li *et al.*, 2013). The stimulatory effects of potato root exudates to *S. subterranea* resting spore germination might also be explained by their organic chemical composition.

Characterization of the root exudate metabolome can assist in gaining a better understanding of plant-microbial interactions. The choice of techniques to be used in metabolomic studies is important to achieve unbiased and successful characterization of a metabolome. Metabolite analysis of aqueous samples, including root exudates, generally uses gas or liquid chromatography which is usually coupled to mass spectrometry (MS). Gas-chromatography is more suitable for volatile compounds, whilst liquid-chromatography (LC) is commonly used for non-volatiles. Recently, new improvements

have been made to LC techniques, particularly for detecting polar, hydrophilic compounds. An improved hydrophilic interaction ultra-performance liquid-chromatography mass spectrometry (HILIC UPLC-MS) approach has been used to simultaneously identify more than 100 analytes from grapefruit extracts in less than 30 min at detection levels as low as 5 ng/ml (Gika *et al.*, 2012).

The present study examined metabolomes of potato root exudates to gain insight into their metabolite composition, and to determine if specific chemical constituents stimulate *S. subterranea* resting spore germination. The bioassays were performed in a soil-less *in vitro* system (Fornier *et al.*, 1996) coupled with microscopy, and the targeted characterization of compounds in the root exudates was achieved using a HILIC UPLC-MS methodology (Gika *et al.*, 2012) This work advances the understanding of the biology of *S. subterranea* resting spore germination and provides new insights into the pathogen's chemical ecology. Furthermore, the primary metabolic composition of potato root exudates is outlined, expanding known metabolite constituents. Potato genotypes used in this study have importance in potato breeding programs and in commercial production. Hence, the metabolite profile may compliment other "omics" approaches for potato improvement.

4.3. Materials and Methods

4.3.1. Potato Root Exudate Collection

Root exudates were collected from deionized water extracts of tissue-cultured potato (*Solanum tuberosum*). Four potato cultivars that varied in their known resistance to *S. subterranea* diseases were used. Cultivars 'Agria' and 'Iwa' are highly susceptible to both tuber and root disease, whilst cvs. 'Russet Burbank' and 'Gladiator' have moderate to strong resistance to tuber infection and moderate resistance to root infection (Falloon *et al.*, 2003, Falloon *et al.*, 2016). Preparation and collection of potato root exudates were performed aseptically. Potato tissue-cultured plantlets were grown in potato multiplication medium (MS salts and vitamins, 30 g/L of sucrose, 40 mg/L of ascorbic acid, 500 mg/L of casein hydrolysate and 0.8% agar, pH 5.8) under a 16 h photoperiod using white fluorescent lamps (65 μmol/m^2/s) at 22 °C (Wilson *et al.*, 2010b). After 2 weeks, plants were gently uprooted from the media, their roots were washed with sterile deionized water, blotted dry on sterile tissue paper, then placed in a sterile polycarbonate

bottle containing 20 ml of fresh sterile deionized water (instrument conductivity at 0.059 μS/cm). For exudate collection plants were incubated in the water solution under the same light and temperature regime as before for varying incubation periods.

Firstly, to confirm the capability of potato root exudates to stimulate resting spore germination and to assess possible influences of cultivar, a series of nine bioassays comparing 32 individual potato root exudates was conducted. Either two ('Gladiator' and 'Agria') or all four cultivars were incubated in the water solution for 7 d prior to collection and testing. Then for analysis of root exudate composition and association with stimulation of resting spore germination a further 12 exudate solutions were collected. Exudates from all four cultivars were collected following 2, 7 and 18 d incubation in the water solution. All exudate solutions were stored in sterile Falcon tubes at -20 °C, in the dark, until used. There were no signs of microbial contamination in the root exudate solutions prior to use. Unless otherwise stated, all chemicals used in this study were sourced from Sigma-Aldrich (St. Louis, MO).

4.3.2. Inoculum Preparation

The *S. subterranea* inoculum (resting spores) was prepared from powdery scab-affected tubers, collected from Devonport, Tasmania, Australia (41.17 °S, 146.33 °E). Diseased tubers were washed with running tap water for 1-2 min, soaked in 2% sodium hypochlorite (White King, Pental Products Ltd Pty, Melbourne, Australia) for 3 min, quickly rinsed, and air-dried. Lesions were excised using a scalpel, dried for 4 d at 40 °C, ground by mortar and pestle, and stored at 4 °C until used. The inoculum was approximately 1 y old when used. Inoculum contained approximately 6,900 sporosori/mg, as determined by suspending 0.1 g of inoculum in 10 ml water and quantification using light microscopy.

4.3.3. Resting Spore Germination Assay

Presence of motile zoospores in the test solutions indicated resting spore germination Fornier *et al.* (1996). Briefly, 1 mg of inoculum (6,900 sporosori) was suspended either in 1.5 ml of root exudate, individual compound solutions (0.1 mg/ml), or in deionized water (control) solutions, each in a 2 ml microcentrifuge tube covered

with aluminum foil. Tubes were incubated for 30 d at 15–18 °C. At 3-7 d intervals, 35 µl from each treatment was subsampled. The number of motile zoospores in the subsample was determined with a DM 2500 LED microscope (Leica Microsystem, Wetzlar, Germany) at 200X magnification, scanning in an inverted "S" manner within a 22 mm x 22 mm area of each microscope-slide. *S. subterranea* zoospores were identified by morphology (Kole, 1954) and motility behavior (Merz, 1997). Assessments of each root exudate solution and 43 individual LMWO compounds were repeated, respectively, seven and two times. Each assay consisted of three replications. In the individual chemical bioassay, Hoagland's solution and sterile deionized water were added, respectively, as a stimulant and non-stimulant controls.

Additional bioassays were performed to further validate *S. subterranea* zoospore identity. The first used a modified tomato-bait test Merz (1989). A 100 µl subsample from each test and control solution was transferred into a McCartney bottle containing 5 ml of non-sterile deionized water and a healthy 2-week old tomato plant (cv. Grape). Tomato plants were left in the solution for 24-48 h at 15-18 °C; to allow zoospores to encyst, and were then removed. Roots were washed to remove any resting spores adhering to root surfaces. Each plant was transferred to a new McCartney bottle containing 20 ml of non-sterile deionized water and grown for an additional 2 weeks to allow the development of zoosporangia. This test was repeated, using different zoospore solution sources. Sample roots were then cut, placed on microscope slides, stained with 0.1% Trypan blue for at least 10 min, and zoosporangia (Ledingham, 1935) were examined under a light microscope. In the second test, the number of zoospores successfully attached to *S. subterranea* roots of cv. 'Gladiator' and cv. 'Iwa' host roots was determined. Single, 2 cm long, roots were placed on microscope slides, which were flooded with 70 µl of test solutions and incubated at room temperature for 10 min. Each test was replicated three times. To ensure all roots received a similar exposure to zoospores, the test was performed one root/cultivar/replicate at a time. The number of zoospores attached on the roots were counted microscopically, at 200X magnification, by scanning the whole root.

4.3.4. Phytochemical Analysis

Detection of primary metabolites in potato root exudate solutions was done using HILIC UPLC-MS (Gika *et al.*, 2012). The UPLC assays were performed using a Acquity

UPLC H-class system (Waters, Milford, MA), and MS analyses using a Xevo triple quadrupole MS system (Waters). HILIC separation was done on a 2.1 mm x 150 mm Acquity 1.7 μm BEH amide VanGuard column maintained at 60 °C and eluted with a two-step gradient at 500 μL/min flow rate for 30 min. The mobile phases were composed of A (acetonitrile/water, 95–5 (v/v), 0.1% formic acid. and 0.075% NH_4OH) and B (acetonitrile–water, 2–98 (v/v), 0.2% formic acid. and 0.1% NH_4OH). LiChrosolv-grade acetonitrile was purchased from Merck (Darmstadt, Germany), whilst formic acid (>95%) and NH_4OH were purchased from Sigma-Aldrich. The gradient started with a 4 min isocratic step at 100% mobile phase A, then rising to 28% mobile phase B over the next 21 min and finally to 60% B over 5 min (Gika *et al.*, 2012). The column was then equilibrated for 12 min in the initial conditions. Two cycles of weak and strong solvent washing of the injection system were carried out between injections. The injection volume was 10 μl and the column eluent was directed to the mass spectrometer. Metabolite detection was achieved using selected ion monitoring and an electrospray ionization source was applied operating in both positive and negative ion mode. The parameters in the electrospray were set as follows; capillary voltage, −2.5 kV or 3 kV; cone and desolvation temperatures, respectively, 150 and 400ºC with a desolvation gas flow of 950 L/h and cone flow of 100 L/h. The cone voltage was optimized for each individual analyte. The identity of metabolites was confirmed by detection of the expected [M+H]+ or [M-H]- molecules at known retention indices (Gika *et al.*, 2012) by the analysis of standard chemicals, as both pure standards and spiked into root exudates samples. The identity of amino acid metabolites was also confirmed by tandem-MS multiple reaction monitoring experiments, with the detection of known product molecules arising from selected precursor molecular species (Gika *et al.*, 2012).

4.3.5. Data and Statistical Analysis

Zoospore numbers were converted to counts per 100 μl solution, prior to statistical analysis using SPSS statistical software, ver. 22, (IBM, Armonk NY). An independent T-test and one-way analysis of variance (ANOVA) were performed for data having, respectively, two and more than two treatments. Data containing two factors were analyzed by two-way ANOVA. Multiple comparison of means was carried out using the Fisher's Least Significant Difference (LSD) test at 0.05 level of probability. HILIC UPLC-MS analyses were done using MassLynx XS software (Gika *et al.*, 2012) The limit of

detection *and limit of quantitation* of the analytes were determined at signal-to-noise ratios of 3 and 10 respectively. A hierarchical cluster dendrogram was constructed using the average linkage (between group) method, with binary data measured using the squared Euclidean distance. A pattern (color) map was constructed to represent the distribution (numbers) of the metabolite/compound classes detected in various root exudates.

4.4. Results

4.4.1. *Spongospora subterranea* Zoospore Identity

Motile biflagellate (of unequal length) zoospores, of approximately 4-5 μm diameter, were observed in the majority of test potato root exudates and occasionally in control (sterile deionized water) solution. The presence of the two flagella on each zoospore were best observed at 400X magnification, but observation of zoospore swimming patterns was best viewed at 200X magnification. Other uniflagellate and biflagellate organisms were present, but their numbers were minimal and their movement patterns distinct from those *S. subterranea* zoospore. Figure 4. 1 shows that zoosporangia, indicative of *S. subterranea* infection, developed when test solutions with *S. subterranea* zoospores were exposed to tomato roots. Similarly, zoospores were observed attached to potato roots with a greater number of zoospores (*P=0.008*) attached to roots of the susceptible cv. 'Iwa' (16.67 ± 2.60) than the resistant cv. 'Gladiator' (3.67 ± 0.33; Figure 4.1).

In the second experiment, the duration of incubation of potato roots prior to exudate collection (2, 7, and 18 d) had no statistically significant effect (*P=0.152*) on stimulation of resting spore germination and while cultivar showed a statistically significant effect (*P=0.050*), there were again no obvious consistent trends (Figure 4. 3). The interaction of incubation period and cultivar had a clear statistically significant effect (*P=0.033*), with differences between cultivar most evident in the 18 d incubation samples (Figure 4. 3).

4.4.3. Metabolite Constituents of Various Potato Root Exudates

The HILIC UPLC-MS analysis detected a total of 24 LMWO compounds from potato root exudates (Table 4. 1). These compounds ranged from 104-504 Da molecular weight, and included eight amino acids, one sugar, three sugar alcohols, five organic acids and seven other organic compounds. Detection of these compounds varied among root

exudates. Asparagine, glutamic acid, glutamine, proline, serine, pinitol, choline, trehalose and tyramine were detected in most of the potato root exudates. However, some compounds were uniquely observed in a particular potato cultivar root exudates or collection date. For instance, raffinose, dehydroascorbic and quinic acid, and adenosine were, respectively, present only in 'Iwa', 'Agria', and 'Gladiator' root exudates and histamine was only detected in the exudates incubated for 18 d. The hierarchical cluster analysis revealed that potato root exudates could be divided at 80% similarity into three groups based on the metabolite composition (Figure 4. 4). Cluster 1 included all 'Iwa' root exudates, cluster 2 was composed of mostly 2 and 7 d incubation and all 'Russet Burbank' root exudates, and cluster 3 were mostly 18 d incubation and 'Gladiator' root exudates.

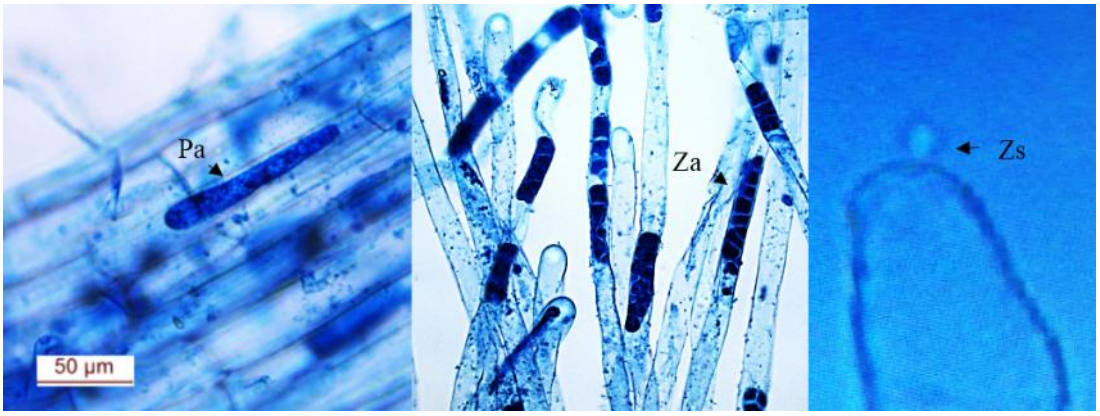

Figure 4. 1. Photomicrographs of *Spongospora subterranea* plasmodia (Pa) and zoosporangia (Za) in tomato roots, and zoospores (Zs) attached on potato root, which validates zoospore identity.

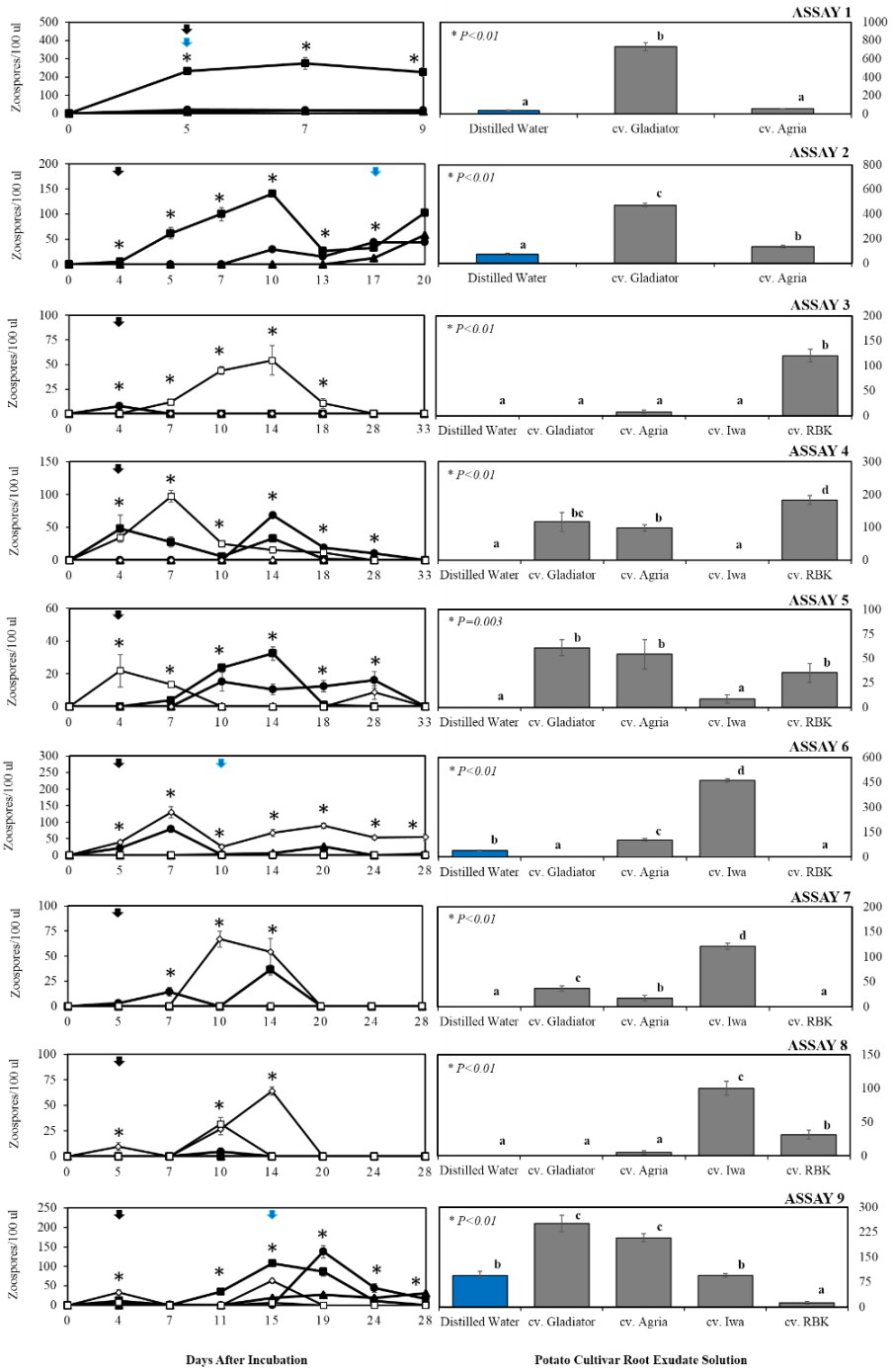

Figure 4. 2. Resting spore germination (zoospore release) of *Spongospora subterranea* as influenced by deionized water (▲) and root exudates of potato cultivars 'Gladiator' (■), 'Agria' (●), 'Iwa' (◇), and 'Russet Burbank' (□). Mean zoospore population at different time intervals (line graph, left) and the accumulated population at the end of incubation period (bar graph, right) are represented. Arrows in black and blue indicate initial zoospore release, respectively, in root exudates and deionized water (control). Vertical bars are standard errors (n=3). Asterisks indicate treatments means, within same day, are statistically different ($p<0.05$) by analysis of variance. Bars with the same letter, within an assay, indicate means are not statistically different ($p<0.05$), Fisher's LSD test.

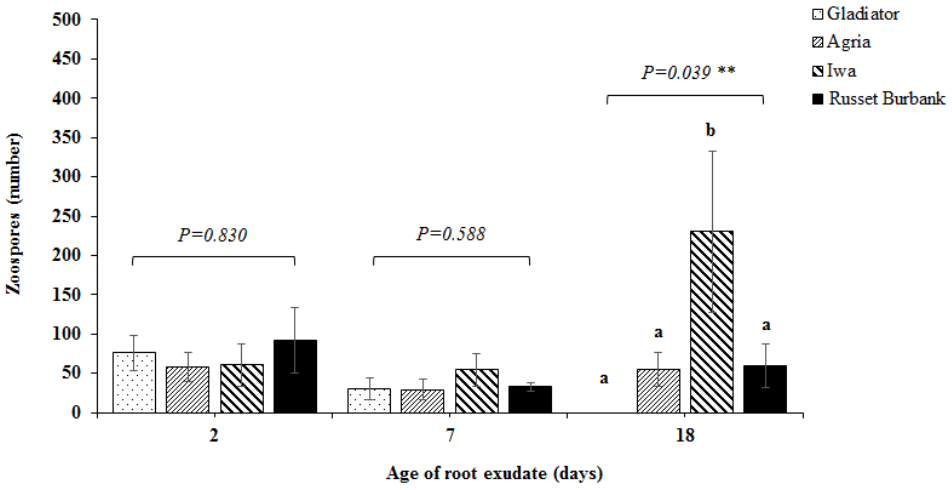

Figure 4. 3. Resting spore germination (zoospore release) of *Spongospora subterranea* as influenced by the age (2, 7, and 18 d old) of potato root exudates. Vertical bars are standard error (n=6). Double asterisk indicates that treatment means, within a group (indicated by a horizontal bar), are statistically different by analysis of variance. Bars, within the 18 d old group, annotated with the same letter are not statistically different ($p<0.05$; Fisher's LSD test). P (incubation time) = 0.152; P (cultivar) = 0.050; P (incubation time x cultivar) = 0.033.

Table 4. 1. Low-molecular weight organic compounds in potato root exudates from different cultivars as detected using the HILIC UPLC-MS technique.

No.	Retention Time (min)	Compound	Compound Class	m/z[a]	Gladiator[c]			Russet Burbank[c]			Agria[d]			Iwa[d]		
					2[b]	7	18	2	7	18	2	7	18	2	7	18
1	1.02	Nicotinamide	Oc	123	+		+				+		+	+		+
2	1.27	Maleic acid [e]	Oa	114				+	+							
3	2.76	Dehydroxyascorbic [e]	Oa	173									+			
4	2.78	Tyramine [e*]	Oc	138	+		+	+	+	+	+		+			
5	2.86	Adenosine [e]	Oc	268		+										
6	4.95	Choline	Oc	104	+	+	+	+		+	+	+	+			+
7	6.75	Pinitol	Sa	192	+		+	+	+	+	+	+	+			
8	6.99	N-acetylcysteine [e*]	Aa	161	+		+						+	+		+
9	7.45	Spermine [e]	Oc	203	+	+	+				+	+				
10	10.39	Histamine	Oc	111			+						+			
11	11.48	Isoleucine [e]	Aa	132			+		+			+	+			
12	12.4	Proline [e]	Aa	116	+	+	+		+		+	+	+	+		
13	13.02	Quinic acid [e]	Oa	190							+					
14	13.36	Malic acid [e]	Oa	132						+	+		+			
15	13.90	Threonic acid	Oa	134						+	+		+			
16	14.48	Lactose	Sg	343		+		+		+	+	+		+		
17	14.52	3-hydroxy proline [e]	Aa	132			+		+			+	+			
18	14.68	Trehalose [e]	Sg	360	+	+	+	+	+	+	+	+	+	+	+	+
19	16.33	Glutamine [e*]	Aa	147	+	+	+	+	+	+	+	+		+		

20	16.39	Serine [e*]	Aa	106	+		+	+	+	+	+	+	+		
21	16.59	Asparagine [e]	Aa	133	+	+	+	+	+	+	+	+	+		
22	17.55	Citruline [e*]	Ac	176				+	+						
23	18.27	Glutamic acid [e]	Aa	147	+	+		+	+	+	+	+	+		
24	19.58	Raffinose [e]	Sg	522										+	

[a] mass/charge ratio of the analyte precursor molecule. [b] The age of the root exudate in days. [c] Cultivar is resistant to powdery scab in the field (Falloon *et al.*, 2003). [d] Cultivar is susceptible to powdery scab in the field (Falloon *et al.*, 2003). Aa, amino acid; Sa, Sugar alcohol; Sg, Sugar; Oa, Organic acid; Oc, Other organic compound. +, compound was detected (present); otherwise, blank (absent). [e] Indicates compounds tested for *S. subterranea* RSG activity. * indicates positive RSG stimulation activity.

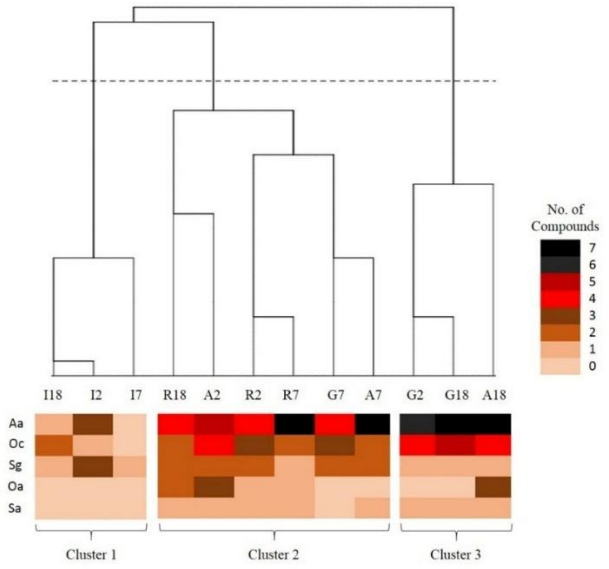

Figure 4. 4. Hierarchical cluster analysis dendrogram (Average Linkage method) and pattern map of the compound class distribution for 12 potato root exudates using the metabolite/compound composition. Abbreviations used; Aa amino acid, Oc other organic compounds, Sg Sugar, Oa organic acid, and Sa sugar alcohol. Potato cultivars 'Iwa', 'Russet Burbank', 'Agria', and 'Gladiator' are denoted, respectively, by I, R, A, G. The numbers 2, 7, and 18 are the incubation period (days) of potato roots in water at the time of exudate collection. The dotted line indicates the 80% threshold point delineating three clusters.

4.4.4. Screening of Metabolites Stimulant to Resting Spore Germination

Testing individual compounds provided a more robust direct relationship of each compound to resting spore germination stimulation (Figures 4. 5 and 4. 6). Stimulation of germination of *S. subterranea* resting spores was chemical-specific (Figure 4. 5). By comparing capacity and timing of zoospore release it was determined that five of the 18 compounds found in potato root exudates and seven of the 25 additional compounds tested were stimulant at 0.1 mg/ml, resulting in zoospore release at least 8 d earlier than the water control (Figures 4.5 and 4.6). L-Glutamine and tyramine had greater effects (*P=0.045* and *P=0.010*, respectively) on resting spore germination than the other compounds tested and the Hoagland's stimulant control. Mean accumulated zoospore numbers in other stimulant LMWO compounds, L-rhamnose, cellobiose, L-aspartic acid, *N*-acetyl cysteine, piperazine, glucoronic acid, L-serine, succinate, L-citrulline and citric acid, were not statistically different from the non-stimulant water control ($P>0.05$) but were deemed stimulant as zoospore release in the presence of these compounds was at least 8 d earlier than in the deionized water control (Figure 4.6).

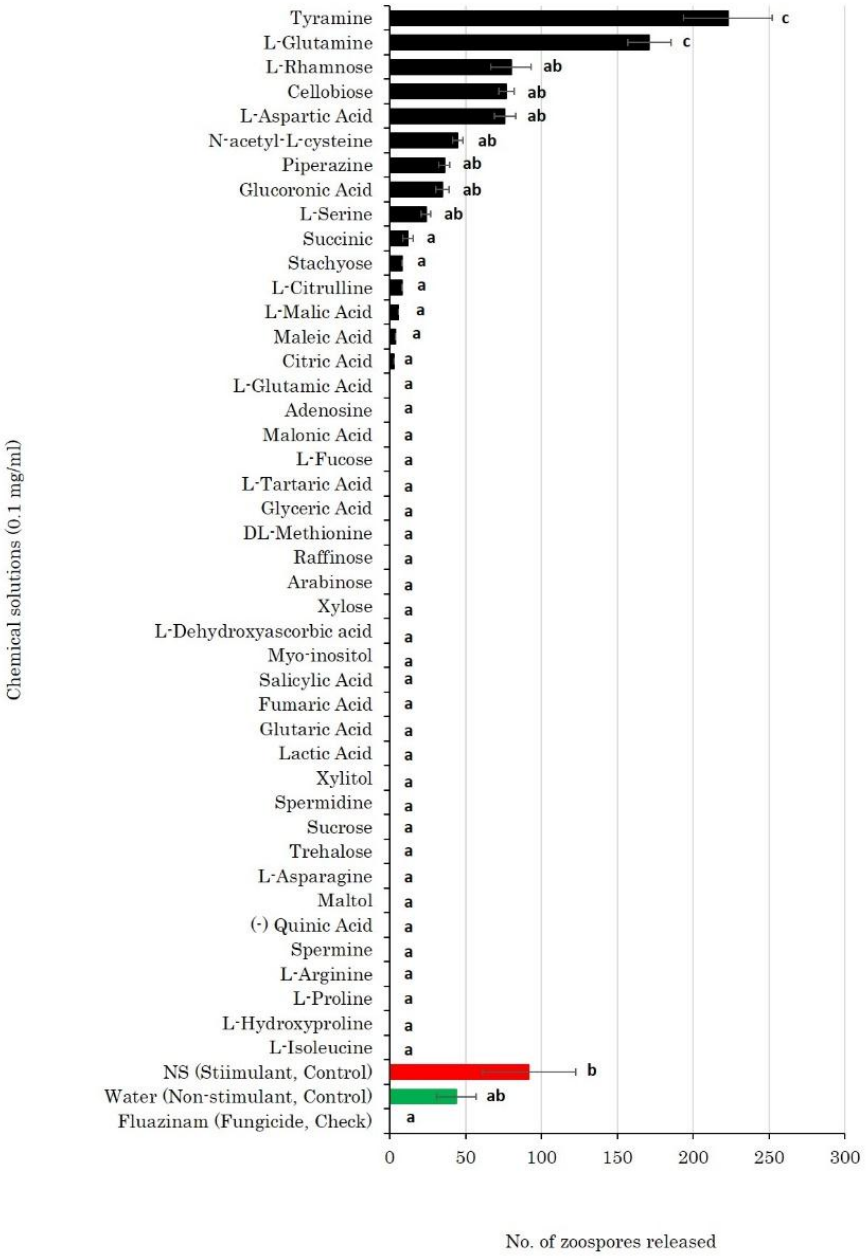

Figure 4. 5. Mean accumulated resting spore germination (zoospore release) of *S. subterranea* as influenced by various organic compounds, the deionized water control or stimulant (Hoagland's) solution. Vertical bars are standard errors (n=6). Treatments with same letter are not statistically significant at $p>0.05$ (Fisher's LSD test).

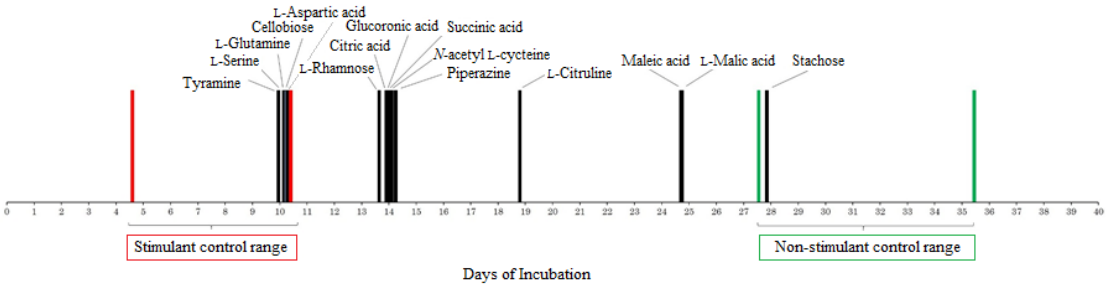

Figure 4. 6. Days to initial *Spongospora subterranea* resting spore germination (zoospore release) in low-molecular weight organic compound solutions (0.1 mg/ml), control (distilled water) or stimulant (Hoagland's) solution. Each bar indicates day of zoospore release.

4.5. Discussion

This study aimed at elucidating, at a metabolite level, the stimulatory effects of potato root exudates on *S. subterranea* resting spore germination. LMWO compounds are the main constituents of plant root exudates and are known stimuli of soil-borne plant pathogens (Schroth & Hildebrand, 1964, Nelson, 2006). In an initial series of *in vitro* bioassays we showed in most instances aqueous potato root exudates stimulated *S. subterranea* resting spore germination, releasing zoospores earlier and in greater numbers than the deionized water control. It was also clear that there was no consistent cultivar effect, with variability between assays likely due to variations in plant physiological condition. Using targeted HILIC UPLC-MS based metabolite profiling (Gika *et al.*, 2012), covering major primary metabolites in a plant biological system, we identified 24 polar LMWO compounds within potato root exudates. The majority of these compounds were amino acids, which is in agreement with other plant species (Carvalhais *et al.*, 2011, Vančura & Hovadík) and from prior metabolomic studies of potato tubers (Roessner *et al.*, 2000, Dobson *et al.*, 2010). The LMWO compounds, however, were not uniformly detected across all potato root exudates, with more compounds detected in the extracts from the cvs 'Agria', 'Gladiator' and 'Russet Burbank' than in the 'Iwa' extracts. Hierarchical cluster analysis of the potato root exudate components separated exudates into three clusters associated with cultivar and exudate incubation period. Similar variations have been observed with both plant and environmental factors influencing release of LMWO compounds (Lynch & Whipps, 1990, Neumann & Romheld, 2007).

Analysis of metabolites from tubers of different *Solanum* spp. indicated individual species could be identified by their metabolic composition (Dobson *et al.*, 2010). Variability of root exudate composition with plant physiological and environmental factors (Carvalhais *et al.*, 2011) suggests these are less useful for biotyping potato cultivars but with knowledge of their biosynthesis it may be possible to investigate genetic and proteome changes in a plant grown under particular conditions. For example, the HILIC UPLC-MS based metabolite profiling (Gika *et al.*, 2012) used in this study could be extended to analyze metabolite change in *S. subterranea*-infected plants to gain further insights into genes expressed by host plants during root infection.

Pathogen germination or activation by host root exudates is a common phenomenon among many soil-borne pathosystem, and this is particularly the case for environmentally-resistant-spore producing pathogens (Schroth & Hildebrand, 1964). The stimulation of resting spore germination by potato root exudates corroborated the findings of earlier reports (Merz, 1993, Fornier *et al.*, 1996). While there could be doubts that the zoospores identified, in this and other studies (Kole, 1954, Fornier *et al.*, 1996, Merz, 1997), may be those of other flagellated contaminant organisms, additional testing showed that the detected zoospores produced zoosporangia after host infection(Falloon *et al.*, 2016), and attached to potato roots in a differential manner associated with known cultivar resistance (Hernandez Maldonado *et al.*, 2012) as would be expected with *S. subterranea*. Furthermore, the initial release of zoospores (4-5 d) coincides with the other reports which have used *in vitro* approaches to examine *S. subterranea* resting spore germination (Fornier *et al.*, 1996, Harrison *et al.*, 1997).

Root exudates from both host and non-host plants and their chemical constituents have been suggested to play a part of the *S. subterranea* pre-infection activation (Kole, 1954, Harrison *et al.*, 1997, Fornier *et al.*, 1996, Merz, 1989) (Chapter 2A). Our study supports these observations. In several other pathosystems individual host specific compounds found within root exudates activate and chemotaxically attract the pathogen spores to a susceptible host plant to facilitate infection (Schroth & Hildebrand, 1964, Bais *et al.*, 2006). Conversely, we have shown that a diverse range of LMWO compounds are stimulatory toward *S. subterranea* resting spore germination. Whilst we show these are produced by the potato host many of these compounds are commonly found in root exudates from other plant species including non-hosts (Vančura & Hovadík, 1965, Carvalhais *et al.*, 2011). Thus we can conclude that resting spore germination is not host-

specific. This is in agreement with studies of the closely related *Plasmodiophora brassicae* (Kowalski, 1996, Ohi *et al.*, 2003, Suzuki *et al.*, 1992, Mattey & Dixon, 2015). It is thus unsurprising that production of stimulatory compounds was not associated with known potato cultivar resistance with cultivar effects quite variable. Rather it is likely that production of stimulatory LMWO compounds will be more strongly influenced by other factors such as the physiological condition of the plant (Neumann & Romheld, 2007). It has been suggested that *S. subterranea* zoospores are likely attracted to root exudates(Kole, 1954) or specific chemical compounds within these exudates (Chapter 2A). This has been well documented in other pathosystems (Bimpong & Clerk, 1970, Islam & Tahara, 2001) and variation in the susceptibility of plants can be associated with variation of chemo-attraction by the zoospores (Zentmeyer, 1961, Deacon, 1996). We provide preliminary data indicating zoospore attachment to potato host roots is affected by resistance of the potato cultivar but to date no studies specifically examining chemotaxic attraction of *S. subterranea* zoospores have been conducted.

Control of potato diseases caused by *S. subterranea* has been partly successful (Falloon, 2008). Planting resistant cultivars has proven the most effective means for powdery scab control, but cultivars lack effective strong resistance to root infection. The use of fungicides provides partial efficiency, reducing disease impact (Falloon, 2008, Thangavel *et al.*, 2015) probably by reducing inoculum pressure or delaying initial zoospore-host interaction. However, there have been general restrictions on the use of synthetic chemicals for disease management due potential health and environmental risks, and on crop toxicity (Aktar *et al.*, 2009). This has resulted in increased efforts to explore alternative chemical and biological control methods. Several investigations used a variety of plant extracts and their active chemical components for control of major vegetable and grain crop diseases (Chitwood, 2002). Others have used organic amendments (Gamliel *et al.*, 2000) and biological control agents to eradicate or reduce inoculum levels and minimize disease outcomes (Janisiewicz & Korsten, 2002), while some continuously test other chemical substances (Martin, 2003). Few of these types of investigations have been applied to *S. subterranea* diseases of potato (Falloon, 2008). The present study identified several root exudate compounds that stimulate *S. subterranea* resting spore germination and application in absence of a host plant could assist in reducing soil inoculum levels by removing viable resting spores (Chapter 2A).

We acknowledge that the tests were done *in vitro,* and that the root exudates were collected from plants grown in sterile aqueous solutions. Compounds detected in this study may differ from those exuded by plants grown under field conditions due to soil environmental and biological factors. Nevertheless, studies using root exudates from *in vitro* cultures provide clear indications of direct interactions of the pathogen with host root exudates due to the absence of potential compounding factors which could affect pathogen behavior (Vranova *et al.*, 2013). Moreover, the main objective of this study was to determine whether compounds released by potato roots influence *S. subterranea* resting spore germination. Identifying physiological and environmental factors that may influence potato root exudation, particularly those influencing the release of chemical-stimulants in potato root exudates, is worthy of further investigation (Chatper 2A).

In conclusion, have demonstrated further support, and provide the first direct evidence, to the previous findings that potato root exudates stimulate *S. subterranea* resting spore germination (Merz, 1993, Fornier *et al.*, 1996). We also showed that these biologically active potato root exudates contain polar LMWO compounds, and that specific compounds present within these exudates and related compounds stimulate resting spore germination. This study offers new knowledge of the LMWO chemical constituents in potato root exudates that directly influence resting spore germination and thus, makes a contribution to knowledge of the pathogen's biology and chemical ecology. Active compounds are known from root exudates of other plants (including non-hosts) which supports the contention that resting spore stimulation is not host specific. Our findings may also be of importance to other root-infecting pathogens of potato that may be influenced by root exudation. From an integrated pest management perspective, the use of non-pesticidal resting spore germination-stimulating chemical compounds to decrease the levels of infective agents (zoospores) during cropping breaks, could provide a new method to manage *S. subterranea* soil inoculum, and potentially decrease the subsequent disease outcomes (Chapter 2A).

Supplementary Table 4. 1. Composition of the Hoagland's solution used in this study.

Chemicals	Final Concentration
Calcium nitrate	253 ppm [a]
Magnesium sulfate	722 ppm
Potassium nitrate	2.3 ppm
Ammonium nitrate	246 ppm
Potassium phosphate	40 ppm
Iron-EDTA [c]	20 ppm
Micronutrients group	
Boric Acid	140 ppb [b]
Pottasium chloride	400 ppb
Manganese sulfate	90 ppb
Zinc sulfate	115 ppb
Copper sulfate	50 ppb
Molybdic acid	16 ppb
Sodium molybdate	22 ppb

[a] parts per million
[b] parts per billion
[c] ethylenediaminetetraacetic acid

Supplementary Table 4. 2. List of potato root exudates metabolites, with SIMs, molecular formulas, monoisotopic masses, precursor and product ions, ionisation polarity, retention time, cone and collision voltages

No.	Rt [a]	ESI [b]	Name	Molecular Formula	Monoisotopic Mass [c]	Precursor Ion	Product Ion	Cone Voltage	Collision Voltage
1	1.0	+	Nicotinamide	$C_6H_6N_2O$	122,048	123,08	96,03	34	16
2	1.3	-	Maleic acid	$C_4H_4O_4$	116,011	114,85	26,98	16	12
3	2.8	-	Dehydroxyascorbic acid	$C_6H_6O_6$	174,016	173,00	113,00	20	12
4	2.8	+	Tyramine	$C_8H_{11}NO$	137,084	138,00	77,00	12	25
5	2.9	+	Adenosine	$C_{10}H_{13}N_5O_4$	267,097	268,00	136,00	20	17
6	5.0	+	Choline	$C_5H_{14}NO$	104,107	104,00	60,00	22	22
7	6.8	-	Pinitol	$C_7H_{14}O_6$	194,079	192,80	100,80	18	15
8	7.0	-	N-acetylcysteine	$C_5H_9NO_3S$	163,030	161,93	83,91	18	8
9	7.5	+	Spermine	$C_{10}H_{26}N_4$	202,216	203,18	129,02	10	4
10	10.4	+	Histamine	$C_5H_9N_3$	111,080	111,98	83,00	4	12
11	11.5	+	Isoleucine	$C_6H_{13}NO_2$	131,095	132,03	69,00	14	18
12	12.4	+	Proline	$C_5H_9NO_2$	115,063	116,03	69,98	15	10
13	13.0	-	Quinic acid	$C_7H_{12}O_6$	192,063	190,70	84,80	16	13
14	13.4	-	Malic acid	$C_4H_6O_5$	134,022	132,86	70,95	20	12
15	13.9	-	Threonic acid	$C_4H_8O_5$	136,037	134,88	74,92	18	12
16	14.5	+	Lactose	$C_{12}H_{22}O_{11}$	342,116	343,10	162,99	12	12
17	14.5	+	3-Hydroxyproline	$C_5H_9NO_3$	131,058	132,00	86,10	20	19
18	14.7	+	Trehalose	$C_{12}H_{22}O_{11}$	342,116	360,15	163,03	14	14
19	16.3	+	Glutamine	$C_5H_{10}N_2O_3$	146,069	147,03	83,97	14	16
20	16.4	+	Serine	$C_3H_7NO_3$	105,043	106,00	60,00	20	14
21	16.6	+	Asparagine	$C_4H_8N_2O_3$	132,053	133,03	73,94	14	14
22	17.6	+	Citrulline	$C_6H_{13}N_3O_3$	175,096	176,03	70,03	12	20
23	18.3	+	Glutamic acid	$C_5H_9NO_4$	147,053	147,97	84,02	14	16
24	19.6	+	Raffinose	$C_{18}H_{32}O_{16}$	504,169	522,22	343,10	10	10

[a] The compounds typical retention time (Rt) in minutes
[b] Electrospray Ionisation (ESI) mode, in which the compound was either protonated (+) or deprotonated (–)
[c] Values in Daltons

Chapter 5. Potato root exudation and release of *Spongospora subterranea* resting spore germination-stimulants are affected by plant and environmental conditions

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5.1. Abstract

Variation in plant and environmental conditions were studied to determine the effect thereof on the exudation of low-molecular-weight organic compounds by potato roots. The results of the phytochemical analyses showed that among the conditions investigated, root vigour, potato cultivar, nutrients in incubation solution and temperature influenced the number and the type of primary metabolites released. Moreover, these conditions influenced our detection of compounds known to stimulate germination of resting spores of the pathogen Spongospora subterranea, causal agent of powdery scab and root diseases of potato. We conclude that changes in plant and environmental conditions can affect the release of specific compounds that stimulate germination of *S. subterranea* resting spores. The impact of the factors affecting potato root exudation on subsequent disease development is discussed.

Keywords: powdery scab, Spongospora root disease, HILIC UPLC-MS, plasmodiophorid, potato metabolism

5.2. Introduction

Plant roots release a substantial quantity of primary metabolites into the rhizosphere, equivalent to 40– 50% of the total amount of carbon fixed by plants (Bais *et al.*, 2006), the majority of which are low molecular-weight organic (LMWO) compounds. These compounds play important roles in interactions between the plant, and other soil biota including soilborne plant pathogens (Schroth & Hildebrand, 1964, Nelson, 1990,

Haichar *et al.*, 2008, Micallef *et al.*, 2009). Exudate compounds may benefit pathogens, promoting their germination, growth, survival, pathogenesis and reproduction, or may be detrimental inhibiting growth or disrupting motility. The composition of primary metabolites produced by plants including those found in root exudates will be influenced by both the plant's genetics and environmental growth condition (Bertin *et al.*, 2003, Neumann & Romheld, 2007). Various factors have been reported to influence the composition of root exudates for many plant species (Neumann & Romheld, 2007). However, very little is known about the primary metabolites released by potato (*Solanum tuberosum* L.) roots and how this is influenced by plant and environmental factors. This knowledge would assist the understanding of the dynamics of potato root phytochemical–soilborne pathogen interaction.

Recently, we have confirmed specific LMWO compounds detected in potato root exudates (L-Glutamine, tyramine, N-acetyl cysteine, L-serine and Lcitrulline) and related compounds (L-rhamnose, cellobiose, L-aspartic acid, piperazine, glucoronic acid, succinate and citric acid) can stimulate the germination of *Spongospora subterranea* f.sp. *subterranea* (Wallr.) Lagerh. resting spores (Chapter 4). *Spongospora subterranea* is a soilborne potato pathogen responsible for the tuber disease powdery scab (Wallroth, 1842) and root diseases (Falloon et al. 2016). Powdery scab results in significant economic loss to producers of seed tubers, fresh market and processing potatoes (Wale, 2000, Wilson, 2016). Root infections can also negatively impact plant productivity and tuber yields (Falloon *et al.*, 2016). There are no single effective management options for control of *S. subterranea* diseases (Falloon, 2008). The use of stimulant compounds to activate (germinate) pathogen resting spores in the absence of a host plant as an inoculum management strategy has been proposed (Chapter 2A). Primary metabolites in potato root exudates have been characterized, and some compounds known to stimulate the resting spore germination (RSG) process have been identified (Chapter 4). However, the conditions or factors which influence the release of the specific germination-stimulant compounds are unknown. This knowledge may provide further insight into the epidemiology of *S. subterranea* diseases. For example, practices such as continuous (mono) cropping enhance the release of root exudates that activate soilborne peanut pathogens (Li *et al.*, 2013). In this study, the influence of plant age, root vigour, root injury, potato cultivar, light, nutrient availability, plant density and growth temperature on the composition of potato root exudates was investigated. We used a targeted metabolomics

approach able to simultaneously screen a large number of polar LMWO compounds commonly found in plant extracts (Gika *et al.*, 2012). Changes in root exudate metabolite profile that affected release of known *S. subterranea* germination-stimulating compounds were noted.

5.3. Materials and Methods

5.3.1. Plant Materials, Growth Condition and Treatments

Tissue-cultured potato (*S. tuberosum*) cultivars 'Desiree', 'Shepody', 'Gladiator', 'Iwa' and 'Russet Burbank' were used in this study. Cultivar 'Iwa' is highly susceptible to powdery scab, root infection by *S. subterranea* and root galling; 'Shepody' is moderately susceptible to powdery scab and highly susceptible to root galling; 'Russet Burbank' and 'Desiree' are moderately resistant to powdery scab, to root infection and to root galling; and 'Gladiator' is very resistant to powdery scab, root infection and root galling (Falloon *et al.*, 2003, Nitzan *et al.*, 2008, Falloon *et al.*, 2016). Plants were maintained in potato multiplication (PM) medium composed of MS salts and vitamins, 30 g/l sucrose, 40 mg/l ascorbic acid, 500 mg/l casein hydrolysate and 8 g/l agar, pH 5.8, and were grown under 16:8 h day:night cycle photoperiod in white fluorescent lamps (65 lmol m2 /s) at 24°C (Wilson *et al.*, 2010b). Four plant physiological and four environmental factors were examined in the study. Unless otherwise stated, plants were grown for 2 weeks in PM medium prior to transfer to sterile deionized distilled water (SDW) for root exudate collection, were grown at 24°C and received 16 h of light in a 24-h cycle, and 'Russet Burbank' was used as the test cultivar. For the plant age factor, two-node potato cuttings were grown for 1, 2 and 3 months in PM medium prior to individual transfer to SDW for exudate collection. For the root vigour factor, each plant's root mass was measured after root exudate collection. Plants with a root mass greater than 90 g were considered to have strong (vigorous) root growth, whilst those less than 20 g were classed as a weak root system. For the root injury factor, roots were either trimmed by removing 2.5 cm from the root tip, using a sterile scalpel prior to transfer to SDW or left intact. For the cultivar factor, the six potato cultivars were assessed. For the light factor, plants were either incubated under the standard light cycle (16:8 h light–dark cycle) or were held in the dark, by encasing plants and their container in aluminium foil, after transfer to SDW. For the effect of nutrients, plants after 2 weeks in the PM media were transferred first into a

Hoagland's solution (HS; Shah *et al.* 2012) for 7 days, then transferred to SDW for root exudate collection or were transferred to a SDW solution for 7 days, prior to transfer to fresh SDW for root exudate collection. Prior to transfer to SDW for exudate collection, roots were carefully washed in SDW several times and blotted dry to remove any remnant HS. For the plant density factor, either single plants or groups of five plants were placed in a single container with SDW for root exudate collection. For the effect of temperature, plants were incubated at 4, 10, 15, 24 or 30°C during root exudate collection. All treatments were replicated three times. Unless otherwise mentioned, each replicate used a single plant. Preparations were all performed in aseptic condition, to avoid microbial and exogenous chemical contamination.

5.3.2. Root Exudate Collection

Root exudates were collected aseptically from the SDW solutions in which the tissue-cultured potato plants were incubated. Plants were lightly uprooted from the media, roots washed with SDW, blotted on sterile tissue paper and prepared based on their respective treatments. Individual or grouped plants were then placed in a polypropylene tissue culture bottle containing 20 ml of fresh SDW (0.059 lS/cm instrument conductivity). Root exudates were sampled 7 days from the date of plant's transfer and stored at 20°C, in the dark until use.

5.3.3. Phytochemical Analysis

Detection of LMWO primary metabolites or compounds in plant root exudate solution was achieved using a hydrophilic interaction ultra-high-performance liquid chromatography–mass spectrometry (HILIC UPLC-MS) procedure derived from Gika *et al.* (2012). Briefly, the UPLC and MS analysis were performed using the Acquity UPLC H-class and Xevo triple quadrupole MS system (Waters, Milford, MA, USA), respectively. The HILIC separation was performed on a 2.1 mm 9 150 mm Acquity 1.7-lm BEH amide VanGuard column maintained at 60°C and eluted with a two-step gradient at 500 ll/min flow rate for 30 min. The gradient started with a 4- min isocratic step at 100% mobile phase A (acetonitrile–water, 95–5 (v/v), 0.1% formic acid and 0.075% NH4OH), then rising to 28% mobile phase B (acetonitrile–water, 2–98 (v/v), 0.2% formic acid and 0.1%

NH4OH) over the next 21 min and finally to 60% B over 5 min (Gika *et al.*, 2012). The column was then equilibrated for 12 min in the initial conditions. Two cycles of weak and strong solvent washing of the injection system were carried out between injections. The injection volume was 10 ll, and the column eluent was directed to the mass spectrometer. Metabolite detection was achieved using selected ion monitoring, and an electrospray ionization (ESI) source was applied operating in both positive and negative ion mode. The parameters in the electrospray were set as follows: capillary voltage, 2.5 kV or 3 kV; cone and desolvation temperatures, 150 and 400°C, respectively, with a desolvation gas flow of 950 l/h and cone flow of 100 l/h. The cone voltage was optimized for each individual analyte. The repeatability of the HILIC UPLC-MS method was confirmed by analysis of standard chemicals, root exudates and root exudates spiked with standard chemicals.

5.3.4. Data and Statistical Analysis

Hydrophilic interaction ultra-high-performance liquid chromatography–mass spectrometry data were analysed using MASSLYNX XS software (Waters, Milford, MA, USA; Gika et al. 2012). The limit of detection and limit of quantitation of the analytes were determined at signal-to-noise ratios of 3 and 10, respectively. Compounds were identified based on their respective m/z ratio and typical retention time in the chromatogram (Gika *et al.*, 2012). Only compounds detected in at least two of the three replicates were validated as 'present' in root exudate solutions. Compounds were designated '1' and '0' for 'present' and 'absent', respectively. Using the binary data, a hierarchical cluster analysis was performed using SPSS statistical software ver. 22, (IBM, Armonk, NY, USA). A dendrogram was constructed using the average linkage (between group) algorithm and the squared Euclidean distance method. A heat map was also constructed to present the distribution of the compound classes detected in various root exudates. The presence of known *S. subterranea* RSG-stimulant compounds (Chapter 4) was ascertained using the binary data and plotted along with the heat map and in the dendrogram.

5.4. Results

5.4.1. Effect of Plant Physiological Conditions on Root Exudation

The potato plant's physiological conditions affected the number of compounds released by potato roots (Table 5. 1). A total of 24 compounds were detected which included amino acids (12), sugar alcohols (1), sugars (9), organic acids (3) and other LMWO compounds (12). Varying plant age produced no clear trend. Two-month-old plants released more compounds than younger or older plants. Trimmed roots released more sugars than intact roots. Plants with stronger (larger) roots released more compounds in total than plants with weaker (smaller) root systems; however, the numbers of sugars released by the strong and weak plants did not differ. Cultivars also varied in their exudate metabolite profiles with Shepody releasing twice as many compounds as other cultivars (Table 5. 1).

5.4.2. Effect of the Environmental Conditions on Root Exudation

Environmental conditions during the plant's growth influenced the release of LMWO compounds in root exudates (Table 5. 2). The total number of compounds released in root exudates from plants grown in the dark was comparable to those receiving 16 h of light. The release of LMWO compounds in plants which received additional nutrition (Hoagland's solution) was greatly suppressed. The plants with HS supplementation produced seven times less LMWO compounds in their root exudates than the no nutrient-supplemented plants, with substantially less amino acids, and no sugars, organic acids and other organic compounds detected. The number (density) of plants within the collection tube did not influence the number of LMWO compounds in the root exudates. Temperature showed no clear trend with the release of LMWO compounds (12 compounds) being greatest when plants were grown at 10, 24 and 30°C, than at 4 and 15°C (8).

5.4.3. Effect of Physiological and Environmental Factors on the Release of Resting Spore Germination-stimulants

Of the 24 LMWO compounds detected, 15 had been previously tested for *S. subterranea* RSG capacity (Chapter 4), and of these, only four (citrulline, glutamine,

rhamnose and tyramine) were identified as RSG stimulants (Tables 5. 1 and 5. 2). Various plant and environmental factors were found to affect release of these known RSG-stimulant compounds. Notably, the stronger root systems were associated with three of the four RSG stimulants whilst weaker roots had none but otherwise, there were no other obvious associations with any specific treatments (Figure 5. 1).

5.5. Discussion

Potato root exudates contain primary LMWO metabolites some of which are stimulatory to RSG of the powdery scab pathogen *S. subterranea* (Chapter 4). Factors influencing potato root exudation are poorly understood, and hence, the factors which affect the release of the known RSG-stimulant compounds are largely unknown. In this study, changes in some plant physiological and environmental conditions during exudate collection were found to affect the total number and distribution of primary metabolites in plant root exudates. In particular, plants of cv. 'Shepody', those with larger (vigorous) roots and those which did not receive nutrient supplementation released a greater number of LMWO compounds than their respective comparator treatments. These conditions also influenced the release of citrulline, rhamnose, tyramine and glutamine, known to be stimulants of RSG of *S. subterranea* (Chapter 4). Previous studies of primary metabolites of potato have primarily examined tubers, because of their economic value (Roessner *et al.*, 2000, Dobson *et al.*, 2010). Compounds released in potato root exudates have received less attention despite their importance in plant growth and productivity and their ability to manipulate the rhizosphere microbiota including pathogens (Schroth & Hildebrand, 1964). Recently, the primary metabolite composition of potato root exudates from 2-, 7- and 18-day-old plants have been characterized (Chapter 4), where it was shown that plant age and cultivar influenced the type of compounds released. The current study found that in addition to plant age and cultivar, other plant and environmental conditions can affect both the number and type of compounds detected in potato root exudates. The effect of these physiological factors and environmental conditions on the release of compounds in root exudates is similar to previous reports from other plant species (Rovira, 1959, Rovira, 1969). For instance, a greater number of sugars were released from trimmed than intact roots. This is observed when cell walls are damaged (Kumar *et al.*, 2004).

Table 5. 1. The influence of plant factors on compounds detected in potato root exudates out of 135 analytes screened by HILIC UPLC-MS.

No.	Compounds[a]	ESI[b]	*m/z*	Rt	Age[c]			Cultivar[d]					Root Injury[e]		Root Vigour[f]	
					1 Mo	2 Mo	3 Mo	Iwa	Des	She	RBK	Glad	Intact	Trim	Strong	Weak
1	3-hydroxy proline [a]	+	132	13,33				+								
2	Alanine	+	90	14,42											+	
3	aminobutyric acid	+	104	13,26	+		+		+	+			+	+	+	+
4	arginine [a]	+	175	19,16	+	+	+		+	+			+	+	+	+
5	asparagine [a]	+	133	15,63			+								+	
6	glutamic acid [a]	+	147	17,35		+	+			+			+	+	+	+
7	glutamine [a*]	+	147	15,17											+	
8	Glycine	+	76	15,51										+		
9	Ornithine	+	133	21,87	+					+	+					+
10	proline [a]	+	116	11,48		+		+	+	+		+	+	+	+	+
11	pyroglutamic acid	-	128	11,27												
12	Tryptophan	+	205	11,24												
SUGAR ALCOHOLS																
13	5 carbon alcohol	-	151	5,18		+										
SUGARS																
14	C12 sugars [M-H] *m/z* 341	-	341	16.5		+										
15	C6 sugars [M-H] *m/z* 179	-	179	8,17	+	+	+			+	+	+		+		+
16	Lactose	+	343	14,48						+			+	+	+	
17	Mannose	-	179	9,14			+			+		+				+
18	rhamnose/fucose [a*]	-	163	2,35	+					+				+	+	
19	Ribose	-	149	2,64		+										
20	sucrose [a]	-	341	13,02		+	+		+	+		+	+	+	+	+

21	trehalose [a]	+	360	16,19	+	+		+		+				+	+	+
22	xylose [a]	-	149	3,97											+	+
ORGANIC ACIDS																
23	glutaric acid [a]	-	131	3,89											+	
24	carboxylic acid [M-H] m/z 87	-	87	4,69		+										
25	oxalic acid	-	89	8,49		+										
OTHER COMPOUNDS																
26	choline	+	176	4,95						+			+	+	+	+
27	citruline [a*]	+	223	16,30				+							+	+
28	guanidine	+	152	7,80											+	
29	histamine	+	88	10,39				+							+	
30	nicotinamide	+	86	1,21												
31	piperidine	+	89	3,82												+
32	putrescine	+	168	19,13									+		+	
33	pyridoxal	+	169	1,38		+										
34	spermidine [a]	+	203	24,30				+					+	+		
35	spermine [a]	+	265	7,45	+	+	+		+	+	+	+	+	+	+	
36	thiamine	-	138	8,78		+										
37	tyramine [a*]	+	268	2,78									+			

[a] compounds previously tested for *S. subterranea* RSG activity (Chapter 4); * positive RSG activity.
[b] electrospray ionisation
[c] Roots incubated in distilled water for 1, 2 or 3 months prior to exudate sampling.
[d] Des (Desiree), She (Shepody), RBK (Russet Burbank), Glad (Gladiator).
[e] Roots were trimmed by 2.5 cm (trim) or left intact.
[f] Roots were large (>90 g fresh weight) or weak (<20 g)

Table 5. 2. The influence of environmental factors on compounds detected in potato root exudates out of 135 analytes screened by HILIC UPLC-MS.

No.	Compounds[a]	ESI [b]	m/z	Rt	Light[c]		Temperature[d]					Nutrients[e]		Plant Density[f]	
					Dark	16 h Light	4 °C	10 °C	15 °C	24 °C	30 °C	No HS	With HS	5 Plants	1 Plant
1	3-hydroxy proline [a]	+	132	13,33							+				
2	alanine	+	90	14,42								+			
3	aminobutyric acid	+	104	13,26	+	+	+	+	+	+	+	+			+
4	arginine [a]	+	175	19,16	+	+		+		+		+	+	+	+
5	asparagine [a]	+	133	15,63								+			
6	glutamic acid [a]	+	147	17,35	+	+				+	+	+		+	+
7	glutamine [a*]	+	147	15,17								+			
8	glycine	+	76	15,51											
9	ornithine	+	133	21,87									+	+	+
10	proline [a]	+	116	11,48	+	+	+	+	+	+	+	+	+	+	+
11	pyroglutamic acid	-	128	11,27				+				+			
12	tryptophan	+	205	11,24								+			
SUGAR ALCOHOLS															
13	5 carbon alcohol	-	151	5,18											
SUGARS															
14	C12 sugars [M-H] m/z 341	-	341	16.5											
15	C6 sugars [M-H] m/z 179	-	179	8,17		+	+	+	+		+			+	+
16	lactose	+	343	14,48	+	+		+		+	+	+			
17	mannose	-	179	9,14			+	+	+						+
18	rhamnose/fucose [a*]	-	163	2,35		+		+			+			+	
19	ribose	-	149	2,64											

20	sucrose [a]	-	341	13,02	+	+	+	+	+	+	+	+		+	+
21	trehalose [a]	+	360	16,19			+	+	+			+			+
22	xylose [a]	-	149	3,97								+			+
ORGANIC ACIDS															
23	glutaric acid [a]	-	131	3,89								+			
24	carboxylic acid [M-H] m/z 87	-	87	4,69											
25	oxalic acid	-	89	8,49										+	
OTHER COMPOUNDS															
26	choline	+	176	4,95	+	+		+		+	+	+		+	+
27	citruline [a*]	+	223	16,30								+		+	+
28	guanidine	+	152	7,80								+			
29	histamine	+	88	10,39	+					+	+	+			
30	nicotinamide	+	86	1,21			+		+						
31	piperidine	+	89	3,82											+
32	putrescine	+	168	19,13	+					+		+		+	
33	pyridoxal	+	169	1,38											
34	spermidine [a]	+	203	24,30	+					+	+			+	
35	spermine [a]	+	265	7,45	+	+	+	+	+	+	+	+		+	
36	thiamine	-	138	8,78											
37	tyramine [a*]	+	268	2,78	+					+					

[a] compounds previously tested for *S. subterranea* RSG activity (Chapter 4); * positive RSG activity.
[b] electrospray ionisation
[c] Roots incubated in distilled water in full dark or 16 h light 8 h dark cycle prior to exudate sampling.
[d] Roots incubated in distilled water at 4 – 30 °C prior to sampling
[e] Roots were incubated in Hoagland's solution (with HS) or distilled water (no HS) for 7 days prior to exudate collection.
[f] Incubation tubes held 5 plants or 1 plant prior to sampling.

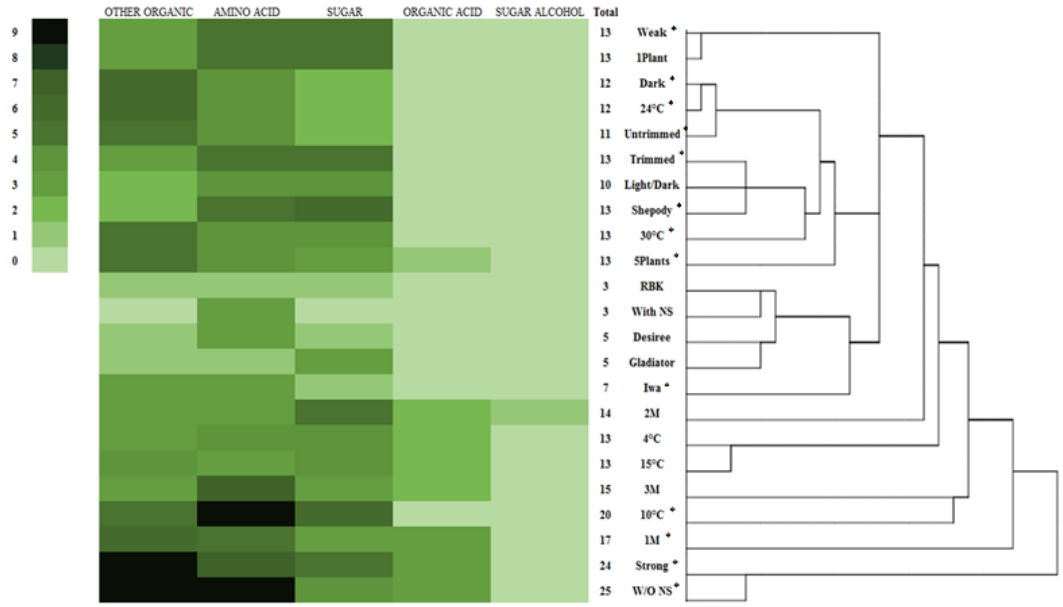

Figure 5. 1. Hierarchical cluster analysis dendrogram (average linkage method) and heat map of the compound class distribution using the metabolite/compound composition in root exudates of potato grown in various physiological and environmental conditions. The distribution of *Spongospora subterranea* resting spore germination-stimulant compounds (Chapter 4) in relation to the dendrogram and heat map is also presented.

To our knowledge, this is the first study of factors influencing the release of compounds in potato root exudates in vitro. Similar to studies on potato tuber metabolism where metabolites could be used as phenotype markers (Dobson *et al.*, 2010), primary metabolite composition in root exudates presented in this study may be used to predict plant's response to different internal and external conditions. Moreover, these primary metabolite profiles may encourage studies on potato soilborne pathogen chemical ecology. It has been shown that root exudates play a critical role as stimulants of *S. subterranea* RSG (Kole, 1954, Merz, 1993, Fornier *et al.*, 1996). And recently, we have shown that potato root exudates contain certain LMWO compounds which are stimulant to RSG (Chapter 4). In this study, we found that the release of some of these stimulant compounds could be influenced by the plant's growing conditions. This implies that these factors help predict the presence of these known stimulant compounds in plants grown in vitro and may affect disease development (Li *et al.*, 2013). Potato roots at all development stages are susceptible to infection (Thangavel *et al.*, 2015), and thus as roots develop, the RSG activation capacity and potential for infection will increase. However, whilst we demonstrate that many of these plant and environmental factors influence root

exudation composition including the release of known *S. subterranea* RSG stimulants, it is important to acknowledge that many of these factors will have additional effects on disease expression through interaction with the pathogen or host plant. For example, temperature has been clearly demonstrated to influence both root and tuber infection (De Boer *et al.*, 1985, Van De Graaf *et al.*, 2005, Van De Graaf *et al.*, 2007).

One important factor identified in this study that affected the release of LMWO compounds was the addition of nutrients. The number of LMWO compounds was greater in plants which did not receive the nutrients (HS) and contained two known RSG stimulants. The higher exudation following incubation in distilled water rather than HS could possibly be attributed to the presence of phosphate substance in HS which can prevent leakiness of cell membranes and release of exudates (Rovira, 1959, Rovira, 1969) although the influence of other compounds cannot be discounted. However, the most important implication to emerge from this finding was the inhibition of the release of known RSG-stimulant compounds in nutrient-supplemented plants. It is thus interesting to speculate whether fertilisers may affect root exudation and indirectly reduce stimulation of *S. subterranea* RSG. Previous studies have shown that high rates of nitrogen applications to fields can increase powdery scab disease (Tuncer, 2002, Shah *et al.*, 2014) although the mechanism for increased disease is not known. Interestingly, detailed glasshouse studies of nutrient effects on potato root infection by *S. subterranea* suggested nitrogen in ammonium form could reduce root infections (Falloon *et al.*, 2014).

There was speculation that cultivars with relative resistance to *S. subterranea* may produce less RSG stimulants than susceptible cultivars. Differences in exudate profiles were found between cultivars; however, we show that resistant cultivars also release the known germination-stimulant compounds, particularly if they have strong root system. This indicates that the initial interaction of the pathogen with its potato host is likely influenced by conditions that affect root exudation. However, this does not mean that the release of RSG-stimulant compounds from a resistant cultivar is necessarily followed by successful host infection or disease expression (Falloon *et al.*, 2003). One of the difficulties in the study of compounds affecting soilborne pathogen activities is distinguishing organic compounds or substances released by roots and those already present in the soil (Rovira, 1969). This study used an in vitro soil-less culture system which allowed robust identification of plant root metabolites in the absence of soil-related confounding factors

(Vranova *et al.*, 2013), and thus more accurately determined the influence of specific plants and environmental factors on root exudation.

Our results support the hypothesis that, under an in vitro system, changes in potato root exudate composition and the release of *S. subterranea* RSG-stimulant compounds is influenced by both plant and environmental factors. Furthermore, we show that whilst cultivar affected root exudate composition, the release of *S. subterranea* germination-stimulant compounds was not associated with cultivar susceptibility to disease. Factors which affected the release of stimulant compounds may also influence disease outcomes; in particular, greater root mass may encourage greater release of RSG stimulants. Further studies examining the role of RSG stimulants in *Spongospora* disease epidemiology may inform development of new disease control tools (Chapter 2A).

Chapter 6. The potential of resting spore germination stimulating-compounds for the management of *Spongospora* diseases of potato

6.1. Abstract

Powdery scab and root galling results from the infection of potato tubers and roots by zoospores of *Spongospora subterranea.* Pivotal to infection is the release of primary zoospores from resting spores present in the soil. Prior studies suggested indirectly that Hoagland's solution (HS) stimulated zoospore release. Here we confirm experimentally that HS does stimulate zoospore release and, in the presence of a susceptible host, will elevate root infection severity. Comparisons of individual components of HS identified that only Fe-EDTA had a stimulatory effect on zoospore release. In a glasshouse trial, we found that adding HS and Fe-EDTA (0.05 and 10 mM) to *S. subterranea*-infested soil for 34 days, in absence of a susceptible host, significantly reduced pathogen content compared to the water only treatment. When treated-soils were subsequently planted with a susceptible potato (cv. Iwa), presence of root galling in plants grown in pots that received Fe-EDTA treatment was half that in plants from the water control, but the difference was not significant. This study suggests that chemical stimulants such as Fe-EDTA can be used to promote early zoospore release and strategic application of such compounds may aid in decreasing soil inoculum which, in conjunction with other strategies, may be beneficial in reducing subsequent disease.

Keywords: *Powdery scab, Spongospora root infection, Fe-EDTA, Hoagland's solution, chemical control*

6.2. Introduction

Powdery scab and root disease, caused by the plasmodiophorid *Spongospora subterranea* (Wallr.) Lagerh., are threats to global potato production (Harrison *et al.*, 1997, Merz & Falloon, 2009). Powdery scab diminishes tuber quality which causes substantial economic losses to fresh market potato industries. It also devalues potato seed crops through the failure to meet seed certification standards (VICSPA, 2007). Processing crops also decrease in value or may be rejected if severely infested (Wilson, 2016). Root diseases

caused by *S. subterranea*, characterised by the formation of zoosporangia and root galls disrupt the normal functions of roots (Lister *et al.*, 2004, Falloon *et al.*, 2004) and contribute to yield loss (Gilchrist *et al.*, 2011, Falloon *et al.*, 2016). In Australia, losses to the processing sector alone have been estimated at A$ 13.4 million per annum or approximately 4% of the gross production value (Wilson, 2016).

Management of *S. subterranea* disease in potato remains a challenge with no single effective control method available (Falloon, 2008). Chemical control has been partly successful in reducing disease incidence and severity but little is known of the effect these fungicides have on inoculum concentration in the soil. Whether effective fungicides are directly biocidal on *S. subterranea* or protect the host from zoospore invasion remains a topic worthy of further investigation. The pathogen persists for many years in the soil, as resting spores or sporosori (conglomerates of resting spores) (Falloon *et al.*, 2011). Upon release from resting spores, motile short-lived zoospores, must locate a susceptible host within approximately 5 hours (Merz, 1997) or perish (Kole, 1954, Karling, 1968).

White (1954) showed that powdery scab incidence and severity could be reduced by growing the alternative host Jimsonweed (*Datura stramonium*) in fields several weeks prior to planting potato. It was hypothesised that planting *D. stramonium* diminished *S. subterranea* soil inoculum by stimulating zoospore release from resting spores. Root infection of *D. stramonium* occurred, however the pathogen was unable to complete its life cycle and produce a new generation of resting spores to replenish soil inoculum (Jones & Harrison, 1969, Qu & Christ, 2006a).

Earlier studies indicated that Hoagland's solution (HS) could stimulate resting spore germination of *S. subterranea* (Ledingham, 1934, Kole, 1954, Merz, 1989, Merz, 1992, Merz, 1997) (Chapter 3). However, no direct measurements of zoospore release were undertaken. Furthermore, the identity of the specific chemical component/(s) of HS that are germination stimulants is not known. If HS stimulates *S. subterranea* zoospore release and the specific stimulant-compound in HS is identified, then HS and the stimulant could be used to induce zoospore release in the absence of the host. This approach, could be used to reduce *S. subterranea* inoculum without the need for growing an alternative host crop (Chapter 2A).

The present study aimed to provide direct evidence for the stimulation of *S. subterranea* resting spore germination by HS and to identify the specific HS component(s)

responsible for the stimulatory effect. Furthermore, we aimed to demonstrate the potential use of stimulant compound for inoculum and disease management.

6.3. Materials and Methods

The laboratory bioassays and glasshouse pot trials were performed in 2014 and 2015 at the laboratory and glasshouse facilities of the University of Tasmania, Tasmanian Institute of Agriculture based in New Town Research Laboratories, New Town, Tasmania, Australia.

6.3.1. Inoculum Preparation

Inoculum was prepared from powdery scab-infected tubers collected from Devonport, Tasmania, Australia (41.17° S, 146.33° E). Potato tubers were washed with running tap water for 1-2 minutes, soaked in 2% bleach solution for 3 minutes, rinsed and air-dried. Lesions were excised, oven-dried for 4 days at 40°C and ground using a mortar and pestle. The inoculum concentration (6,900 sporosori/mg) was determined by suspending 100 mg of powdered inoculum in 10 ml of distilled water and counting sporosori using a haemocytometer.

6.3.2. Effect of Hoagland's Solution on Resting Spore Germination

The effect of Hoagland's solution (HS) on resting spore germination was studied following the method of Fornier (1997) with modification. Dried *S. subterranea* inoculum (1 mg) was placed in a 2-ml microcentrifuge tubes covered with aluminium foil, containing either 1.5 ml HS or deionised distilled water (DW). Eight assays were performed with each assay replicated three times. The microcentrifuge tubes were incubated at 15-18°C and 35 µl subsamples taken at 2-6 days intervals for up to 28 days. Subsamples were placed onto slides, mounted under a 22 x 22 mm cover slip, and examined in an inverted "S" manner with a light microscope at 200X (DM 2500 LED, Leica Microsystem, Germany) to determine zoospore number.

6.3.3. Identification of *S. subterranea* Zoospores

S. subterranea zoospores were identified by observation of distinctive swimming patterns Merz (1992) and zoospore morphology (Kole, 1954, Merz, 1992, Merz, 1997). A bait-plant verification test was also conducted (Merz, 1989). Three-week old healthy tomato (cv. Grape) seedlings were transferred into McCartney bottles containing 5 ml distilled water. A subsample (100 µl) from the HS and water test solutions containing sporosori inoculum were added to the McCartney bottle and incubated for 24-48 hours. Plants were removed from the solution, roots washed carefully and transferred to pathogen-free HS for further incubation to observe root infection development (Ledingham, 1935). Since resting spores initially release zoospores at 4-5 days (Harrison *et al.*, 1997, Fornier, 1997), it was likely that the subsequent root infection can only result from zoospores already in the solution. A second infectivity test measured zoospore encystment on host potato roots. Fine potato roots of cvs. Iwa and Gladiator (2 cm long), susceptible and resistant to powdery scab (Falloon *et al.*, 2003), respectively, were placed separately on a microscope slide, then the slide was added with 70 µl of test solutions. The set-up was left at room temperature for 10 minutes to allow zoospores to establish attachment. The test was replicated thrice. To ensure that all roots received the same time of exposure to zoospores before assessment, the test was performed one cultivar/rep at a time. The number of zoospores encysting on roots was quantified microscopically at 200X.

6.3.4. Effect of Hoagland's Solution on Root Infection

A similar bait-test described above was used to examine the impact of the presence of Hoagland's solution which contained host tomato plant and inoculum. Three-week-old healthy tomato (cv. Grape) plants, grown in the glasshouse, were gently uprooted and roots washed with water to remove adhering soil, before placing the plants into individual vials (30 ml capacity) containing 25 ml HS and 1 mg of dried sporosori inoculum. Vials were placed on a tube-rack and incubated in a growth chamber with 16 hours daylight, 60% humidity at 15-18°C for 4 weeks. Roots were washed, half of the roots cut (middle to bottom) and placed on a microscopic slide and stained with 0.1% trypan blue for 10 minutes and observed by light microscropy at 200X magnification. Presence of zoosporangia were assessed using the severity rating scale: 0 (no infection), 1

(sporadic, zoosporangia covering approximately 1% of the roots), 2 (slight 2-10%), 3 (moderate, 11-25%), 4 (heavy, 26-50%) and 5 (very heavy, >50%). Tomato plants grown in distilled water containing inoculum served as the control treatment. The assay was repeated three times with 3 (Assay 1) and 5 (Assays 2 and 3) replications in each.

6.3.5. Effect of Individual Hoagland's Solution Chemical Components on Resting Spore Germination

To determine which HS component stimulated resting spore germination, each was tested individually in a sporosori-containing solution. The concentrations of each compound tested were equivalent to those found in HS (Table 6. 1). A total of three assays were performed using HS as stimulant control (except assay 1) and DW as non-stimulant control. All chemicals used in this study were sourced from Sigma-Aldrich (USA), except potassium phosphate (AnalR-BDH Chemicals Australia), ammonium nitrate (Prolab, UK), calcium nitrate and potassium nitrate (Chem Supply, Australia).

Subsequently, the effect of six Fe-EDTA concentrations (0.05, 0.5, 1, 2, 5 and 10 mM) on resting spore germination was tested using the method described above. Each assay was replicated three times, with measurements terminated when the zoospore population approached zero.

6.3.6. Glasshouse Pot Trial

A test of the effect of HS and Fe-EDTA on *S. subterranea* DNA in the soil and subsequent disease outcomes were evaluated in a glasshouse pot trial consisting of two control treatments and three chemical treatments (Figure 4). Each treatment was replicated four times with pots arranged in randomized complete block design. A 12-inch pot containing a nursery grade potting mix was amended with 500 mg of sporosori-inoculum, except in the no-inoculum soil treatment (T1). Prior to chemical or water treatments, the quantity of *S. subterranea* inoculum in each pot was determined by thoroughly mixing the soil and collecting 2 gram of soil and testing by quantitative real-time PCR (qPCR). One liter of each chemical or distilled water treatment was added to the surface of the soil with applications repeated six times over 34 days to maintain soil wetness, but avoid water-logged condition. After 34 days from the first chemical treatment, soil subsamples were taken for re-analysis of *S. subterranea* DNA concentration.

The longevity of incubation (34 days) was based on the results obtained from the previous bioassays where upon no zoospores were observed in stimulant containing solutions after this time-period. The average glasshouse daily temperatures during the trial period was 21°C.

To assess the effect of the chemical soil treatment on inoculum potential of treated soils, a 3-week old tissue-cultured plant of the *S. subterranea* susceptible cultivar Iwa was transplanted into each treated pot. Disease evaluation was done after three-months from transplanting and roots were assessed for presence of galls using a standard severity key (http://www.spongospora.ethz.ch/LaFretaz/scoringtable galls.htm) where; 0=no infection, 1=1-3 galls, 2=4-10 galls, 3=>10 galls measuring <10 mm, and 4 =>10 galls measuring >10 mm.

6.3.7. qPCR Testing of Soil Samples

To quantify *S. subterranea* DNA content from soil samples, 210 pg of potato DNA was added to each sample and total soil DNA was extracted using a commercial DNA isolation kit (PowerLyzer® PowerSoil® DNA Isolation Kit, Mobio Laboratories Inc., USA) according to the manufacturer's protocol. DNA was subsequently quantified using a Qubit® 2.0 Fluorometer (Invitrogen, USA). qRT-PCR analysis targeting the ribosomal internal transcribed sequence (ITS) gene region of *S. subterranea (*Hernandez Maldonado *et al.*, 2012) was performed by following the protocol of Thangavel *et al.* (2015). Known concentration of *S. subterranea* DNA was used to construct standard DNA curve and this was used to calculate the DNA concentration in the soil samples using the thermal cycler's internal computing software. To control experimental and technical variation, the relative expression of *S. subterranea* was normalised against the cox1 reference gene of potato expressed from the spiked potato DNA using the Pfaffl (2001) model used for relative quantitation of in real-time PCR. The concentration of *S. subterranea* DNA in the soil samples was averaged (three technical replicates) and expressed in pg/g of soil. A composite soil sample of the control and a test treatment (with *S. subterranea,* 10 mM Fe-EDTA-treated) were also sent to the South Australian Research and Development Institute (SARDI) for confirmatory soil DNA testing for *S. subterranea* DNA content.

Table 6. 1. Chemical composition of the Hoagland's solution used in this study.

Chemicals	Final Concentration
Calcium nitrate	253 ppm*
Magnesium sulfate	722 ppm
Potassium nitrate	2.3 ppm
Ammonium nitrate	246 ppm
Potassium phosphate	40 ppm
Iron-EDTA***	20 ppm
Micronutrients group	
Boric Acid	140 ppb**
Pottasium chloride	400 ppb
Manganese sulfate	90 ppb
Zinc sulfate	115 ppb
Copper sulfate	50 ppb
Molybdic acid	16 ppb
Sodium molybdate	22 ppb

*parts per million, **parts per billion; ***ethylenediaminetetraacetic acid

6.3.8. Data Analysis

The number of zoospores were converted to zoospores per 100 µl prior to analysis of variance using IBM® SPSS® Statistics (Version 22, Armonk NY). Tukey's HSD test was used for multiple comparison of means at 5% significance level. Mean difference in zoospore host-settlement were compared using an independent t-test analysis. The mean pathogen soil DNA concentration, before and after chemical incorporation, and accumulated zoospore population were log-transformed prior to analysis by paired and independent t-test analysis, respectively.

6.4. Results

6.4.1. Zoospore Identity in Solution

The identity of released *S. subterranea* zoospores in the test solutions were confirmed by observation of morphological features (size, presence of two flagella), swimming pattern, and uniformity (the pathogen population outnumbered contaminants) within the solution. Pathogenicity bioassays in tomato and potato confirmed the presence

of infective *S. subterranea* zoospores resulting in zoosporangial infection (tomato) or the settlement of zoospores on root tissue (potato). In the potato bioassay, the relatively susceptible cultivar Iwa had 80% more zoospores attached on roots than the relatively less susceptible cultivar Gladiator ($P = 0.008$) (Table 6. 2).

Table 6. 2. *S. subterranea* zoospore root-settlement preference in potato.

Potato Cultivars	Powdery scab resistance (Falloon et al. 2003)	Mean zoospores settled on host roots
Gladiator	Very Resistant	3.70 ± 0.3
Iwa	Very Susceptible	16.7 ± 2.6
P value		0.008*

* mean difference is highly significant at *P<0.01* based on independent t-test analysis. Values are mean ± standard error (n=3).

6.4.2. Effect of HS on Zoospore Release

Microscopic assessment revealed that in seven of the eight assays, HS stimulated significantly greater zoospore release than DW ($P<0.001$) (Figure 6. 1). In all assays where zoospores were detected in DW treatments, including the single assay where DW released more zoospores than HS, the release of zoospores occurred earlier in HS (4-7 days) than in DW (7-10 days). Zoospore release patterns were similar in all assays and treatments with numbers peaking 3-7 days after being first observed followed by a rapid decline.

6.4.3. Effect of HS on Root Infection

Incubation of *S. subterranea* sporosori in HS was shown to increase the severity of zoosporangia infection in tomato roots compared to the distilled water control treatment (Figure 6. 2). The effect was highly significant in Assay 1 (*P=0.007*) and Assay 3 (*P<=0.001*), whilst a similar but non-significant ($P=0.065$) trend was recorded in Assay 2.

6.4.4. Specific HS Component Stimulating Zoospore Release

Studies on individual components of HS showed that only Fe-EDTA provided a similar stimulatory effect on zoospore release to that of HS (Figure 6. 3). No zoospores were released in the control solution (DW) in any of the three assays. Germination of resting spores occurred at a similar rate and release pattern for all Fe-EDTA concentrations (0.05 – 10 mM). No zoospores were observed in any treatment after 34 days.

6.4.5. Effect of Pre-Plant Chemical Treatment on *S. subterranea* DNA Concentrations in Potting Media

HS and Fe-EDTA, incorporated as pre-plant soil treatments, significantly reduced *S. subterranea* DNA concentration in the soil after 34 days incubation compared to the control treatments (Figure 6. 4). The reduction of DNA concentration was highest in HS amended soil (89% of the initial DNA concentration), followed by 10 mM Fe-EDTA (88%), and 0.05 mM Fe EDTA (83%), however, these three treatments were not significantly different from each other ($P=0.615$). Confirmatory results from an independent laboratory (SARDI, South Australia) revealed the same reduction level of *S. subterranea* DNA (89%) after Fe EDTA (10 mM) soil treatment. *S. subterranea* DNA was not detected in no-inoculum soils with calculated values equivalent to that of the no-template control.

6.4.6. Effect of Pre-Plant Chemical Treatment on Root Galling Severity

Potato plants grown in soils treated with 10 mM Fe-EDTA had a lower severity (1.5) of root galling compared to plants grown in the dry (2.25) and distilled water only (3.0) treated soils, however, the differences were not significant ($P>0.05$) (Table 6. 3).

Table 6. 3. Effects of different moisture and chemical treatments of potting media inoculated with *Spongospora subterranea* on the severity of root galling on potato cv. Iwa.

Treatments	Root Galling Score [1]
Uninoculated soil	0.00 ± 0.00 [a]
S. subterranea inoculum, Dry	2.25 ± 0.48 [b]
S. subterranea inoculum, Water	3.00 ± 0.00 [b]
S. subterranea inoculum, Fe-EDTA (10 mM)	1.50 ± 0.65 [ab]
S. subterranea inoculum, Fe-EDTA (0.05 mM)	2.25 ± 0.48 [b]
S. subterranea inoculum, Hoagland's Solution	3.00 ± 0.00 [b]

[1] Determined using standard severity scoring (http://www. spongospora.ethz.ch/LaFretaz/ scoringtablegalls.htm) where: 0 is no galls; 1 has 1 to 2 galls; 2 has 3-10 galls mostly small (<2mm), 3 has >10 galls, most in clusters, and, 4 has many galls, regularly distributed. Values are actual mean ± standard error, n=4.

* Means are different at the 0.05 level of significance using Tukey's HSD. Means followed by the same letter are not significantly different ($P>0.05$).

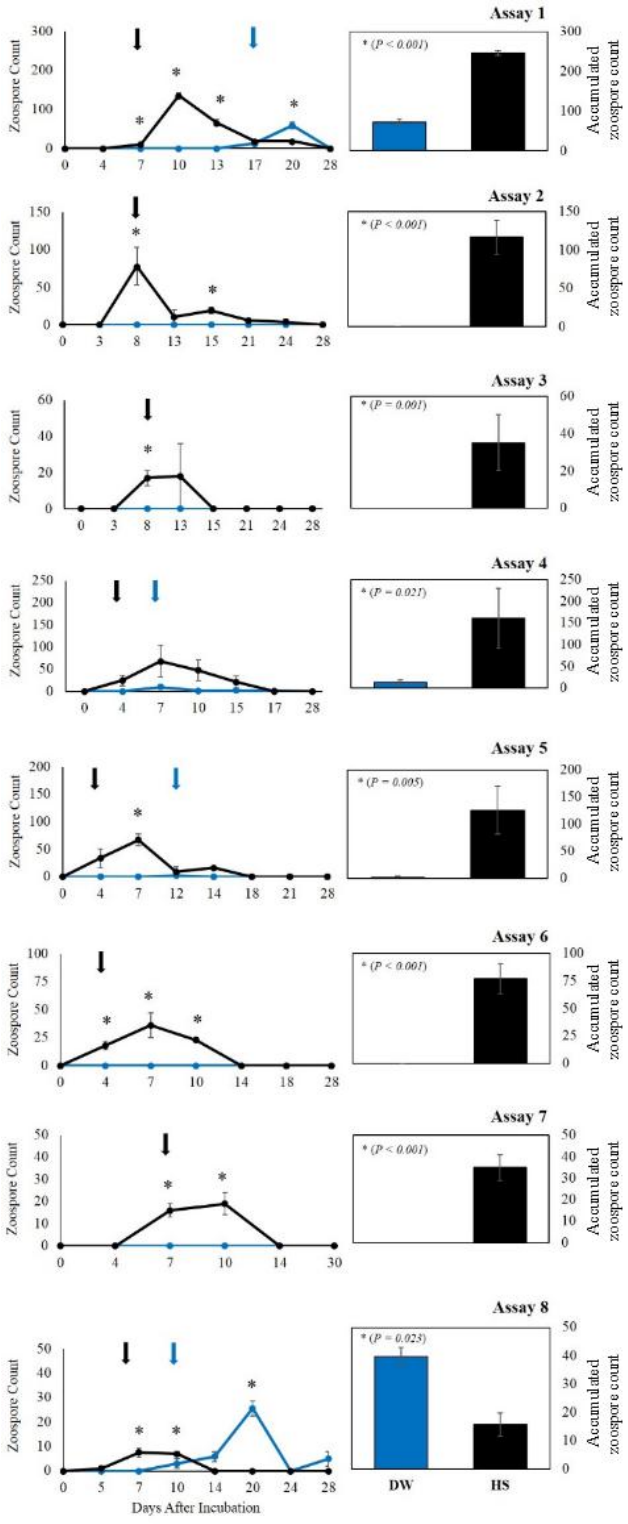

Figure 6. 1. The effect of distilled water (DW) and Hoagland's solution (HS) on abundance of S. subterranea zoospores at time intervals (left) and on accumulated zoospore population up until the end of incubation period (right). Arrows indicate the initial date of zoospore release in DW and HS. Verticals bars are standard errors. Asterisk indicate treatments means are significantly different ($p<0.05$). Mean values in line graph are average of three replicates

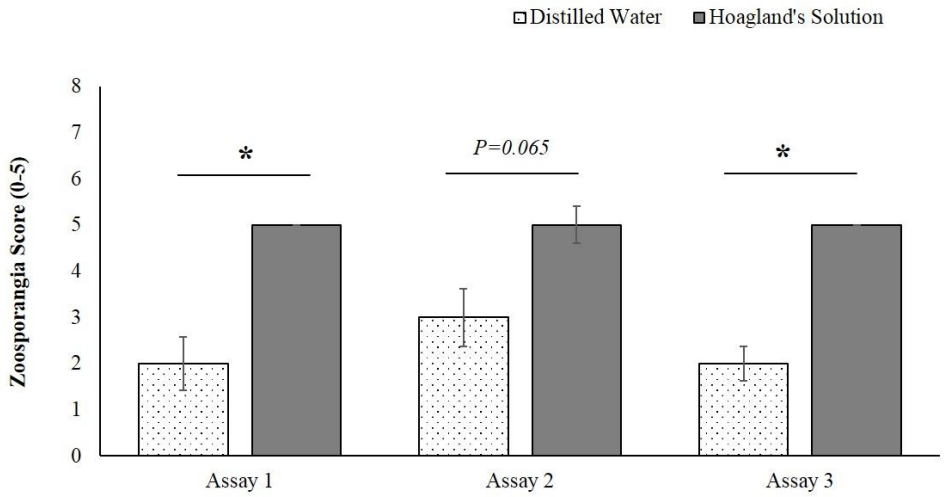

Figure 6. 2. Mean zoosporangia root infection severity (see text for description) in tomato (cv. Grape) after four weeks' incubation with *S. subterranea* sporosori in either distilled water or Hoagland's solution. Verticals bars are the means standard error.

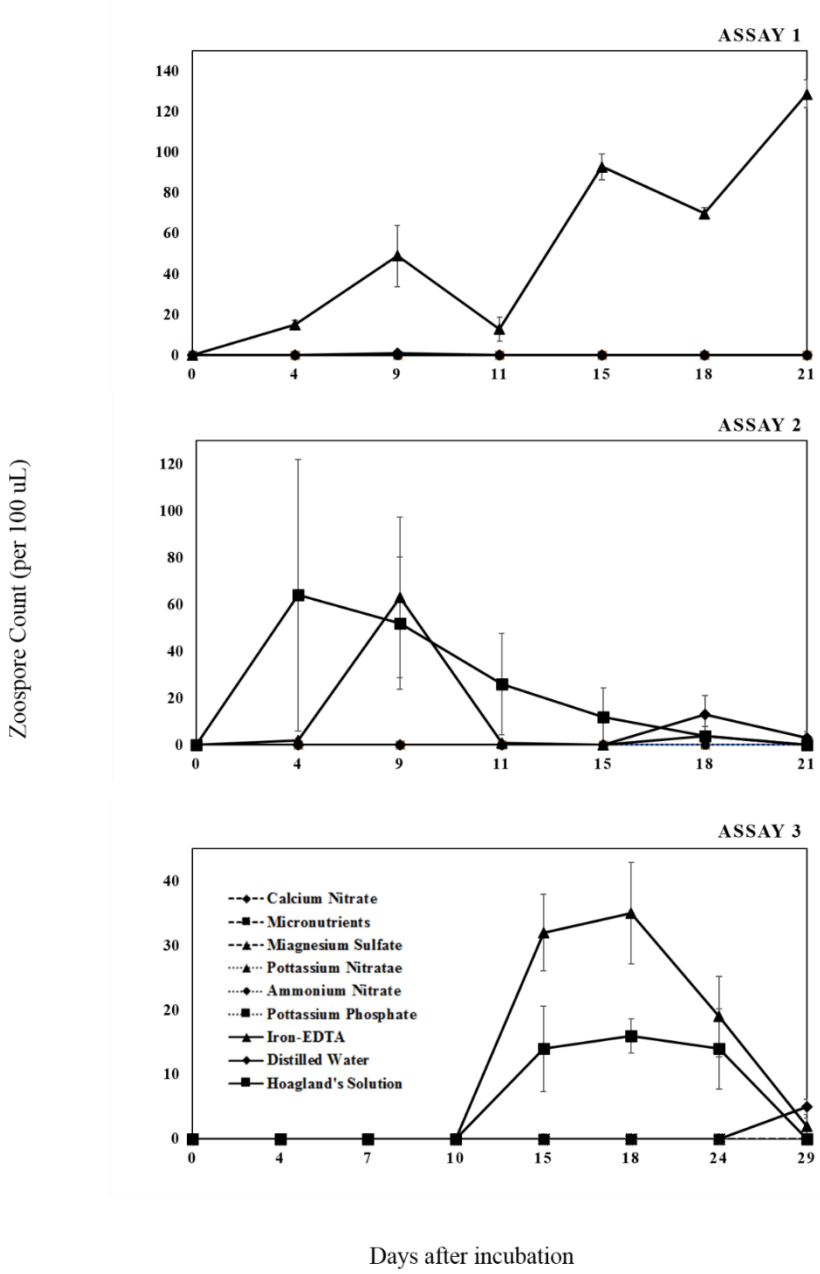

Figure 6. 3. *S. subterranea* zoospore release in solutions containing individual components of Hoagland's solution (see Table 1 for components). Verticals bars are standard errors (n=3).

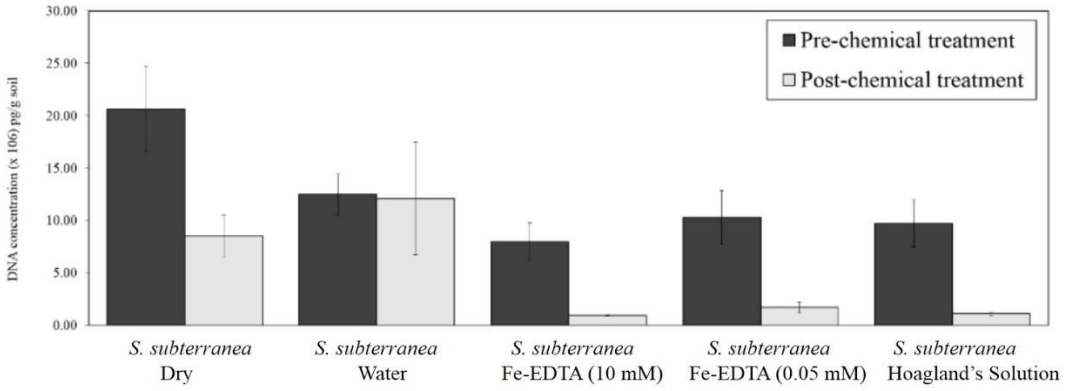

Figure 6. 4. *S. subterranea* DNA concentration (actual) in soil before and after treatment. Treatment solutions incorporated into potted mix soil six times within 34 days. Treatment "dry" indicate the soils were not moistened. Initial (pre-treatment) and final (post-treatment) DNA concentration quantitation was done the day before application of chemical treatments and after 36 days from first treatment, respectively. Verticals bars are standard errors.

6.5. Discussion

Previous studies examining root infection by *S. subterranea* indicated that Hoagland's solution probably stimulated *S. subterranea* resting spore germination (Ledingham, 1934, Kole, 1954, Merz, 1989) (Chapter 3). This study provided direct experimental support of these findings but further identified that Fe-EDTA is the active constituent of Hoagland's solution responsible for stimulating resting spore germination. Hoagland's solution and Fe-EDTA treated with sporosori inoculum stimulated greater and earlier zoospore release than distilled water. We found that addition of HS to an aqueous suspension of sporosori inoculum in the presence of a susceptible host plant exacerbated root infection, and that addition of HS and Fe-EDTA to soil infested with *S. subterranea* sporosori in absence of a host plant substantially reduced *S. subterranea* levels. Whilst subsequent planting into these treated soils did not provide significant reduction in root disease, we note the original inoculum level was very high and the cultivar used was very susceptible. Fe-EDTA treatment in soils with low inoculum levels and planting of a resistant cultivar may provide greater disease reduction potential. Thus, this study provided evidence that resting spore germination stimulants could be used to provide practical benefit for inoculum management of *S. subterranea*-infested soils (Chapter 2A).

The behaviour of *S. subterranea* zoospores in response to chemical compounds has not been widely investigated due to difficulties in working with an obligate parasite,

acquiring uncontaminated zoospore suspensions and challenges in zoospore identification. Using a combination of morphological (Kole, 1954, Merz, 1992, Harrison *et al.*, 1997, Fornier *et al.*, 1996) and behavioural diagnostics (Merz, 1997), we were able to identify *S. subterranea* within non-sterile solutions. Validation of zoospore identity was provided using a bioassay tests (Merz, 1989) and in assessment of zoospore settlement behaviour on potato roots. In the latter assay, we show that persistence to tuber disease expressed by cv. Gladiator (Genet *et al.*, 1995) is reflected in reduced zoospore settlement on roots providing evidence for an extension of cultivar resistance to root tissues (Genet *et al.*, 1995, Falloon *et al.*, 2003).

Ledingham (1934), Kole (1954), and Merz (1989) observed that resting spores suspended in HS increased severity of zoosporangia within host plant roots. Merz (1992, 1997) suggested this was due to enhanced stimulation of zoospore release. Recent study (Chapter 3) showed that this was the case. Our study confirmed that HS enhanced zoospore release and provided the first detailed comparative data on zoospore numbers. We showed HS stimulated both greater number and earlier release of zoospores compared to DW alone. When studying disease dynamics, Van de Graaf *et al.* (2005, 2007) found no significant differences in root infection severity between plants grown in soils with varying levels of sporosori. This suggests that it is not the quantity of inoculum but rather the timing of plant-pathogen interaction (Thangavel *et al.*, 2015) that is critical, particularly for a polycyclic pathogen where disease progresses in an exponential manner once initiated (Berger, 1977, Pfender, 1982, Thangavel *et al.*, 2015). Thus, early release of zoospores in HS, would bring forward the initiation of root infection which would explain the greater severity of root infection in response to HS treatment (Ledingham, 1934, Kole, 1954, Merz, 1989). This finding supports our and others observations that varying loads of sporosori in the soil are not correlated with the severity of root infection (Van De Graaf *et al.*, 2005, Van De Graaf *et al.*, 2007). This suggests that any disease control option targeting soil inoculum in a polycyclic disease, as is the case with *S. subterranea*, will need to reduce pathogen levels to very low levels to provide practical disease management benefits. Therefore, further optimisation of the chemical treatment is required and may include more frequent and longer exposure times prior to crop planting.

HS has been shown or suggested to stimulate zoospore release in other plasmodiophorids, including *Plasmodiophora brassicae* (Asano *et al.*, 2000, Friberg *et al.*, 2005)

and *Polymixa betae* (Ahm & Buchenauer, 1993). The similarity of response to HS suggests that plasmodiophorids may share a similar mechanistic response to stimulation of resting spore germination. It is unknown whether Fe-EDTA is similarly active against these other pathogens.

There is a dearth of information on the chemical ecology of *S. subterranea* and the specific chemicals that influence zoospore release and taxis (Chapter 2A, Chapter 4). Although HS has been routinely used to create a pulse of *S. subterranea* zoospore inoculum for biological and pathological studies (Merz, 1989, Merz, 1992, Merz, 1997, Falloon *et al.*, 2011), the resting spore stimulatory activity of the individual chemical components of HS have not been reported. Our study was able to identify a single component that stimulated zoospore release, Fe-EDTA. In Australia, De Boer *et al.* (2015) reported reduction in soil pathogen levels, disease incidence and powdery scab severity, and these effects were presumed to be due to Fe-EDTA enhancing potato resistance to the pathogen. In contrary, our study showed that this was likely due to reduction of (primary) inoculum in the soil. From a disease control perspective, using low concentrations of non-biocidal chemicals against soil-borne pathogens is desirable as it can avoid plant toxicity and harm to other beneficial soil microbes. While this initial glasshouse trial identified that Fe-EDTA can reduce *S. subterranea* soil DNA levels, it was not able to eradicate the pathogen. This may partly explain the lack of significant differences between Fe-EDTA and control treatments, when assessing root galling.

Adding chemical compounds that stimulate the growth or release of infective agents (e.g. zoospores), in the absence of the host, is an effective approach in reducing pathogen inoculum levels and subsequently disease incidence and severity. For instance, Davis *et al.* (2007) showed that application of garlic powder and diallyl disulfide in the field reduced the population of viable sclerotia of *Sclerotium cepivorum* (cause of white rot of onion and garlic) to less than 10%. Most recently, this approach was used by Mattey and Dixon (2015) to prematurely germinate *P. brassicae* resting spores. In *S. subterranea,* White (1954) indirectly showed that this approach works in the field, using decoy plant Jimsonweed (*Datura stramonium*). Stimulating pathogen germination, in host's absence, to reduce inoculum population has been also shown to control outbreaks of human disease-causing pathogens (Nerandzic & Donskey, 2013). Here, we have identified Fe-EDTA as a stimulant of *S. subterranea* resting spore germination. Previous experiments in New

Zealand and Australia showed reduction in incidence and, in some trials, severity of powdery scab after soil and foliar application of Fe-EDTA (De Boer *et al.* 2015). Therefore, Fe-EDTA could be suitable for reducing *S. subterranea* inoculum levels in the soil and the subsequent disease outcomes in the field (Merz & Falloon, 2009, Wilson, 2016, Falloon, 2008, Braithwaite *et al.*, 1994, Falloon *et al.*, 1996, Thangavel *et al.*, 2015). The work reported here may offer a new potential control approach that could reduce *S. subterranea* soil inoculum levels and disease outcomes. However, further studies on the feasibility of this approach under field condition, cost-benefit analysis of using this approach, the effect of the chemicals on non-targeted soil microorganisms and host plants, and impact on soil chemistry are necessary.

Chapter 7. Discussion, Summary and Future Research

7.1. General Discussion

Either as disease in potato roots or in tubers, *Spongospora subterranea* infection is a major impediment to global potato production (Merz & Falloon, 2009, Falloon *et al.*, 2016, Wilson, 2016) (Chapter 1). Disease control is problematic with Falloon (2008) noting current control measures are unlikely to "give complete control of powdery scab [and root infection] particularly where *S. subterranea* inoculum levels are high on seed tubers or in soil". Therefore, practical ways to control and manage high soil inoculum levels (Chapter 2A) are essential to augment current disease control measures (Chapter 2B, Falloon 2008). This may only be possible by firstly expanding our understanding of the factors which affects resting spore survival and germination (Falloon, 2008). This thesis contributes to this search. The studies described and presented here enhances the knowledge of resting spore biology (Chapter 3, Chapter 4, Chapter 6), presents new information on pathogen chemical ecology (Chapter 4, Chapter 5, Chapter 6) and advances understanding of the epidemiology (Chapter 6) of *S. subterranea* diseases. The findings of this thesis provide new information of the most critical event in the pre-infection stage of *Spongospora* disease pathogenesis, resting spore germination. Taken all together, this study proposes a "germinate/exterminate" approach (Wheeldon *et al.*, 2008) as a potential new control strategy for the management of *S. subterranea* inoculum (Figure 7. 1) and subsequent disease. Incorporating this approach will augment and strengthen other current management programs for *Spongospora* diseases (Falloon, 2008).

7.1.1. Biology of *S. subterranea* Resting Spore Survival

Resting spores of plasmodiophorid pathogens survive for many years in the soil in the absence of their hosts. For *S. subterranea,* direct resting spore examination revealed that they could survive for at least 4-5 years as dried inoculum (de Boer, 2000, Chapter 3). Through observations of natural rotations, Merz and Falloon (2009) suggest that the pathogen may survive in the soil for more than 10 years. The ability to persist in the soil for a long period has been a major barrier in the control of *Spongospora* disease. For instance, crop rotation had limited impact on disease control (Sparrow *et al.*, 2015). The

longevity of survival was believed due to dormancy of the resting spores (Harrison *et al.,* 1997, Merz, 2008, Merz & Falloon, 2009). Although many resting spores germinate in the presence of water, others do not. Chapter 3 indicated that *S. subterranea* resting spores exhibits both stimuli-responsive (exogenous) and non-responsive (constitutive) dormancy (Cochrane, 1974, Deacon & Deacon, 2005, Feofilova *et al.*, 2011). The former germinate when stimulus, e.g. plant root exudates, are present. The latter remain dormant in the presence of a specific stimulus and require physical changes before reverting to stimuli responsive spores. Direct examination of resting spores after and during exposure to stimulants could further elucidate the dormancy characteristics of *S. subterranea.* Nevertheless, understanding of the type of dormancy that *S. subterranea* undergoes has a significant role in long-term inoculum reduction measures. With an understanding of the soil elements/factors which leads to rapid resting spore germination of these dormant spores, inoculum levels in the soil could be reduced (Falloon, 2008, Chapter 2A).

7.1.2. Biology and Ecology of *S. subterranea* During Resting Spore Germination

Despite the ability of *S. subterranea* to survive in the soil without the host, resting spores germinate quickly in the presence of a susceptible host (Ledingham, 1935, Kole, 1954). Chapter 3 and Chapter 4 suggest that this is likely because *S. subterranea* resting spore germination is a "host-triggered" reaction (Halvorson, 1959, Foster & Johnstone, 1990). In the presence of a host (Chapter 3), germination can occur which then leads to root infection. This triggered mechanism of resting spore germination is also displayed by *P. brassicae* (Macfarlane, 1970, Kageyama & Asano, 2009, Friberg *et al.*, 2005), a related pathogen. Some non-host plants can also trigger *S. subterranea* resting spore germination, but do not lead to root infection due to host incompatibility. This indicates that factors common to host and non-hosts plants, e.g. root exudate phytochemicals, are likely involved during the resting spore germination stage.

The germination of *S. subterranea* resting spores by root exudates was postulated 62 years ago by Kole (1954). Merz, as part of his PhD work, conducted several experiments by using root infection as an indicator. He concluded that the response of *S. subterranea* was non-host specific (Ueli Merz, *personal communication,* 2014). A study by Fornier *et al.* (1996) supported the findings of Merz, directly demonstrating greater release

of zoospores in root exudates than in distilled water. Harrison *et al.* (1997) also observed zoospore release in water using light microscopy. However, it was questioned whether the stimulation of resting spore germination could be exacerbated by susceptible cultivars (Chapter 2A), as occurs in other pathosystems (Rovira, 1959, Schroth & Hildebrand, 1964). Chapter 4 indicated that cultivar reaction to the disease had little effect on *S. subterranea* resting spore germination. Hence, the effect of host root exudates and non-host root exudates on resting spore germination is possibly stimuli-specific. This means that root exudation may have little effect on the variation of cultivar response to infection. Root exudates are composed of various chemicals and are known to influence microbial activities (Bais *et al.*, 2006). Chemicals in the root exudates of host and non-host plants are highly likely to be resting spore germination stimuli (Chapter 4).

Most indications of *S. subterranea* interaction to its environment have been measured by host infection. For instance, to study the effect of stimulants on resting spore germination, White (1954) used a decoy plant to trap zoospores. To measure the effect of heating, bait-plants were used to assess root infection (Kole, 1954, Merz, 1989). However, studies have indicated that with careful assessment of zoospore morphology and swimming behaviour, the germination of resting spores through release of zoospores can be directly examined. Harrison *et al.* (1997) and Fornier *et al.* (1996) observed *S. subterranea* zoospores based on its morphological features, whilst Merz (1992) took note of the swimming patterns to distinguish *S. subterranea* from biflagellate contaminants within the solution. Because of its obligate biotrophic nature (Karling, 1968), *S. subterranea* requires a living host-plant to multiply and is therefore non-culturable in synthetic media. Thus, there is no "pure" culture since the source of inoculum will always contain considerable number of contaminants. Therefore, studies that involves zoospore identification have to explore all possible verification methods to ensure robust identification of a *S. subterranea* zoospore. Chapter 4 and Chapter 6 utilised two additional diagnostic tests, in addition to zoospore morphology (Kole, 1954, Merz, 1992, Merz, 1997) and swimming behaviour (Merz, 1992). The first test used a healthy bait-plant (tomato cv. Grape) and exposed zoospore suspension for at most 48 hours, then removed the plants from the zoospore suspension to a fresh (no-inoculum) solution. Because of the short exposure time to host-roots, only zoospores already in the solution could possibly infect bait plants. The presence of zoosporangia was therefore indicative of *S. subterranea* (Ledingham, 1935). However, care should be considered when interpreting this data as resting spores can still

release zoospores. To overcome this, a control solution containing resting spores but without zoospores, should be included always. Another verificatory test is to expose the zoospores to roots of resistant and susceptible cultivars. Chapter 4 and Chapter 6 observed greater number of zoospores attached on susceptible host (Falloon *et al.*, 2003). This zoospore attachment procedure was noted to correlate well with known tuber resistance phenotypes. Although the two additional tests can be useful, zoospore morphology (Kole, 1954, Merz, 1992, Merz, 1997) and swimming behaviour (Merz, 1997) remains fundamentally important and they should always be benchmarks in identifying *S. subterranea* zoospores.

7.1.3. Chemical Ecology of *S. subterranea*

The germination of *S. subterranea* resting spores by chemical elements has not received much attention, until recently (Chapter 4 and Chapter 6). This was partly due to difficulties in producing and identifying suspension of *S. subterranea* zoospores (Merz & Falloon, 2009). Consequently, the understanding of the *S. subterranea* resting spore germination had been previously compared to those of other plasmodiophorid species, e.g. *P. brassicae* (Macfarlane, 1970). Although the plasmodiophorids have some similar features (Braselton, 2001), the behaviour in response to soil environment conditions can differ. For example, *P. brassicae* responds well to soil pH changes but this is not apparent in *S. subterranea* (Falloon, 2008). Resting spores of *P. brassicae* can be stained with chemicals allowing simpler assessment of spore dormancy (Tanaka *et al.*, 1999, Donald *et al.*, 2002), but *S. subterranea* lacks this characteristic (Balendres MA, Tegg RS, Wilson CR, *unpublished work*) due to its anatomical orientation (Falloon *et al.*, 2011). These variable observations indicate that the interaction of *S. subterranea* to chemical elements in the environment might also differ with that of other plasmodiophorid species, and this has prompted a separate investigation (Chapter 2A).

To identify chemical factors involved during the germination of likely dormant *S. subterranea* resting spores (Chapter 3), Chapter 4, Chapter 5 and Chapter 6 undertook a metabolic and chemical dissection in potato root exudates and Hoagland's solution, respectively. Chapter 4 revealed that several low-molecular weight organic (LMWO) polar compounds were contained in potato root exudates. L-glutamine, tyramine, cellobiose, L-rhamnose and aspartic acid at (0.1 mg/ml) have comparable stimulatory effect to the

stimulant control solution. The release of known stimulant compounds was influenced by host physiology and growth conditions (Chapter 5). Some of these compounds may be present in non-host plant root exudates which can also stimulate resting spore germination (Merz, 1993, Fornier *et al.*, 1996). The nature of the compounds (LMWO, polar) stimulating *S. subterranea* resting spore germination was related to those of the compounds stimulating the germination of *P. brassicae* resting spores (Suzuki *et al.*, 1992, Mattey & Dixon, 2015). This may indicate that if a zoospore can respond to a chemical cue from a root exudate, the factor(s) which caused the zoospore's response, e.g. presence of chemical-binding receptors, are likely common in these two pathogens. However, the specific compounds *S. subterranea* responds to (Chapter 4) differ from those of *P. brassicae* (Ohi *et al.*, 2003).

Hoagland's solution has been previously shown to stimulate *S. subterranea* resting spore germination, resulting in greater root infection. Merz (1992, 1997) directly demonstrated this effect by showing pulse of zoospores which were released in the Hoagland's solution after 10 days. But prior to the study in Chapter 6, the compound in Hoagland's solution responsible for the stimulatory effect on resting spore germination was largely unknown. Chapter 6 identified Iron-ethylenediaminetetraacetic acid (Fe-EDTA) as the stimulant component (0.05 to 10 mM) of Hoagland's solution. Fe-EDTA, when used based on its mode of action against *S. subterranea*, could potentially reduce the infective resting spores in the soil and subsequent disease.

7.1.4. Ecological and Epidemiological Role of Resting Spore Germination Stimulant

Environmental conditions favourable to resting spore germination enhances powdery scab and root infection severity (Merz & Falloon, 2009). These favourable conditions are primarily involved in the release and motility of zoospores (Kole, 1954). For example, presence of water signals resting *S. subterranea* spores that conditions are favourable for germination (Harrison *et al.*, 1997) and zoospores movement towards the host for infection. Solutions containing stimulant compounds result in greater and earlier release of zoospores from resting spores (Chapter 4 and Chapter 6). Early release of zoospores can result in greater root infection than later release (Thangavel *et al.*, 2015). Ledingham (1935), Kole (1954) and (Merz, 1989) have indicated and demonstrated the

effect of stimulant conditions on root infection. Chapter 6 revealed that zoosporangia infection scores of plants grown in Hoagland's solution was greater than in plants grown in the distilled water control solution.

Zoospores are short-lived and, upon release, have to find a compatible host (Karling, 1968), otherwise, it perishes. Using this knowledge, a stimulant solution could be used to manipulate the germination of resting spores and in the absence of plants will deprive zoospores of a compatible host. In Chapter 6, the stimulant chemical solutions were incorporated into the soil for 34 days without compatible host plants. Fe-EDTA and Hoagland's solution significantly reduced *S. subterranea* soil DNA levels. There was also a slight reduction in root gall scores in plants grown in the chemically-treated soil compared to the control pots. There may be several options that could improve the efficacy of this disease management approach. For example, extending the time of chemical treatment or increasing the concentration of the chemical-stimulants, if the chemical's concentration is not toxic to the host plant. Together, when stimulant compounds are used with the host plants, root infection is enhanced. However, when stimulant compounds are used to treat soil without host plants, it can reduce the inoculum levels in the soil. This latter approach is similar to the "germinate/exterminate" strategy (Wheeldon *et al.*, 2008) used for *Clostridium difficile,* a human pathogen.

7.1.5. "Germinate/exterminate", a potential inoculum management approach

Plasmodiophorids spend most of their life in the soil, as resting spores (Braselton, 1995, Karling, 1968, Neuhauser *et al.*, 2010). However, survival may be interrupted by various factors/elements (physical, biological, or chemical) in the soil-environment. Germination in the presence of a susceptible host allows the pathogen to continue its growth within host-plant and the infection subsequently produces new generation of resting spore-inoculum. Germination in the absence of a host, however, is lethal to released zoospores. The latter is advantageous from a disease control perspective (Donald & Porter, 2014, Chapter 2A).

Kole (1954) and Merz (1989) showed in a greenhouse study, that heating soils at 40ºC, which contains *S. subterranea* resting spores can induce resting spore germination. A limitation of this approach, however, is the practicality of a large-scale field treatment. If

this is possible, the temperature at which soil-heating operates could also be lethal to other beneficial soil microbes and fauna (e.g. beneficial insects, arthropods). The use of decoy crops has also been demonstrated to reduce powdery scab infection of subsequent potato crop (White, 1954). This approach has not been widely used, probably because its control mechanism has not been fully elucidated or due to the impracticality and cost of growing a decoy crop that have no economic importance (e.g. Jimsonweed). There have been studies on the biological (antagonistic microbes) factors influencing *S. subterranea* survival and germination (Hoyos Carvajal *et al.*, 2008, Gilchrist *et al.*, 2009, Restrepo Duque *et al.*, 2009, Nakayama & Sayama, 2013). Although it has progressed, biological control of *S. subterranea* is still in its infancy stage where further refinement and research are required (Chapter 2B).

Wheeldon *et al.* (2008) termed "germinate/exterminate", a control approach used to reduce the inoculum (spores) of the human pathogen *C. difficile.* Other studies have adopted this approached and re-termed it as "activate to eradicate" (Nerandzic & Donskey, 2013). The essential criteria for this approach are; 1) the pathogen exhibits dormancy, 2) presence of stimulant compounds 3) and the stimulation of germination of dormant spores should be done in the absence of the host. The primary aim of the stimulation is to activate or germinate spores that do not respond to favourable conditions (Feofilova *et al.*, 2011, Nerandzic & Donskey, 2013). When spores germinate without its host, the pathogen is deprived of nutrients and growth. But this requires timing and therefore knowledge of the pathogen's life cycle is important. For instance, the length of survival of the pathogen after germination is critical. Or, the length of germination process once stimulants are introduced. This thesis proposes that the "germinate/exterminate" approach has potential for the management of *Spongospora* disease in potato (Figure 7.1). The essential criteria of the 'germinate/exterminate" approach were met; Chapter 3 revealed that dormant spore characteristics were exhibited by *S. subterranea,* Chapter 4, Chapter 5 and Chapter 6 showed that non-hazardous chemical stimulants of *S. subterranea* resting spore germination are available and Chapter 6 have shown, under controlled condition, that these stimulants can be used as pre-plant treatments. If future field evaluation underpins the efficacy of the "germinate/exterminate" method, this approach could be a potential strategy for managing high levels of soil inoculum. This approach will augment other disease control measures for *Spongospora* disease. A similar approach was used by Davis *et al.* (2007) to control and reduce the sclerotia levels of *Sclerotium cepivorum,*

causal agent of onion and garlic white rot. Mattey and Dixon (2015) recently proposed a similar concept to control high inoculum levels of the clubroot pathogen, *P. brassicae*.

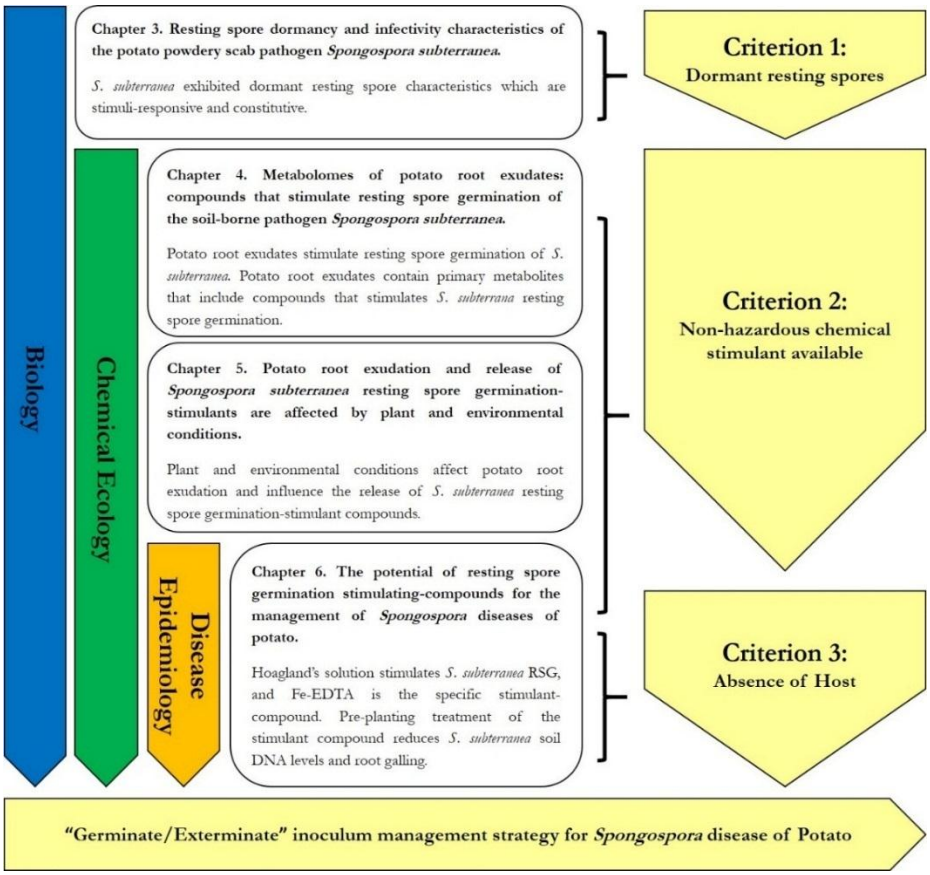

Figure 7. 1. Fitness of "germinate/exterminate" approach in managing *S. subterranea* soil inoculum.

7.2. Summary

This thesis expands our understanding of the resting spore germination biology and chemical ecology of *S. subterranea* (Figure 7. 2). Specifically,

1. Resting spores remained dormant under dry and wet conditions. These resting spores respond to moisture, heat and presence of host roots. However, a

proportion of resting spores are stimuli-responsive and non-responsive (Chapter 3).

2. Potato roots exudates may constitute compounds, which are stimulants to *S. subterranea* resting spore germination (Chapter 4). Potato root exudation is influenced by plant physiology and environmental factors which also affects the release of the known stimulant chemical-compounds in root exudates (Chapter 5).
3. Hoagland's solution contains Fe-EDTA which stimulates resting spore germination. Germination stimulant compounds can exacerbate root infection if host is present during the stimulation. However, germination stimulant compounds can reduce the pathogen's DNA levels in the soil and decrease root galling scores when used as pre-plant (at least a month) treatment (Chapter 6).
4. The 'germinate/exterminate' approach can be potentially used as a new inoculum and disease management strategy (Chapter 7).

The advances in understanding of the biology and chemical ecology of *S. subterranea* resulted in the development of a working framework for the "germinate/exterminate" strategy for *S. subterranea* inoculum management and disease control. This knowledge will encourage further field investigation, to examine the feasibility of the germination stimulant compounds as a chemical control strategy for potato diseases caused by *S. subterranea.*

7.3. Future Research

Spongospora subterranea remains a major threat in global potato-crop production. The pathogen continues to increase the losses to growers and the potato industry (Wilson, 2016). So far, there is no single effective control strategy (Falloon, 2008) and few provides good and long term control for inoculum in the soil (Chapter 2A, Chapter 2B). In this study, we have gained knowledge of the resting spore biology (Chapter 3) and chemical ecology (Chapter 4) of the pathogen. The knowledge is critical in developing control strategies aimed at specific events of the pathogen's disease cycle. The knowledge presented here encourages further investigation of potential chemical control approaches for potato diseases caused by *S. subterranea.*

Despite the use of a qualitative and targeted metabolomic approach, this thesis revealed suites of chemicals *S. subterranea* responds to, during resting spore germination (Chapter 4). However, further investigation of other potential "host-specific" compounds could be valuable. Compounds that are unique to the host may have greater effect on the stimulation of resting spore germination. Quantitative characterisation will also assist in determining the role of the known stimulant compounds. These known stimulant compounds offer a new perspective for inoculum management and this should encourage further investigation. Determining whether these known stimulant compounds also attracts zoospores (chemotaxis) will be important (Figure 7.3, Chapter 2A). Furthermore, the understanding of the potato metabolism may assist in elucidating the resistance genes involved by understanding the changes in the metabolic pathways during *S. subterranea* infection.

The response of *S. subterranea* to several organic compounds released by root exudates indicates that *S. subterranea* undergoes chemical-specific triggering mechanism. Of the compounds tested as pure chemicals, only a few were stimulatory to resting spore germination (Chapter 4). It was first notioned that these compounds were likely released more in susceptible plants, but Chapter 5 revealed that plant and environmental conditions had greater effect on the release of the stimulant compounds. This therefore raised questions whether field practices may have had indirect effect on disease development. Of note, nutrients had an impact in the root exudation of potato *in vitro* (Chapter 5). Investigation of the role of soil nutrition on host root exudates is needed.

Resting spore germination chemical-stimulants presents a novel and cost-effective approach of decreasing *S. subterranea* soil-inoculum (Chapter 6) and therefore provides opportunity to replace current pesticide control practices. The "germinate/exterminate" approach (Chapter 7), however, will have to be tested under more realistic field conditions to determine its commerical feasibility. These further investigations will examine factors which could affect the stimulant-compounds efficacy in the field and what might be needed for this approach to significantly reduce *S. subterranea* inoculum.

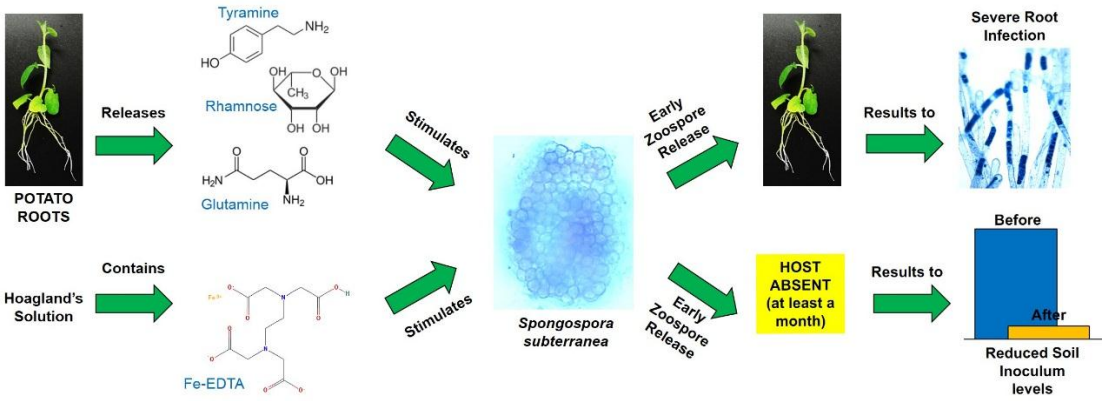

Figure 7.2. Sources and identity of chemical stimulants and stimulant's effect on soil inoculum and disease outcomes.

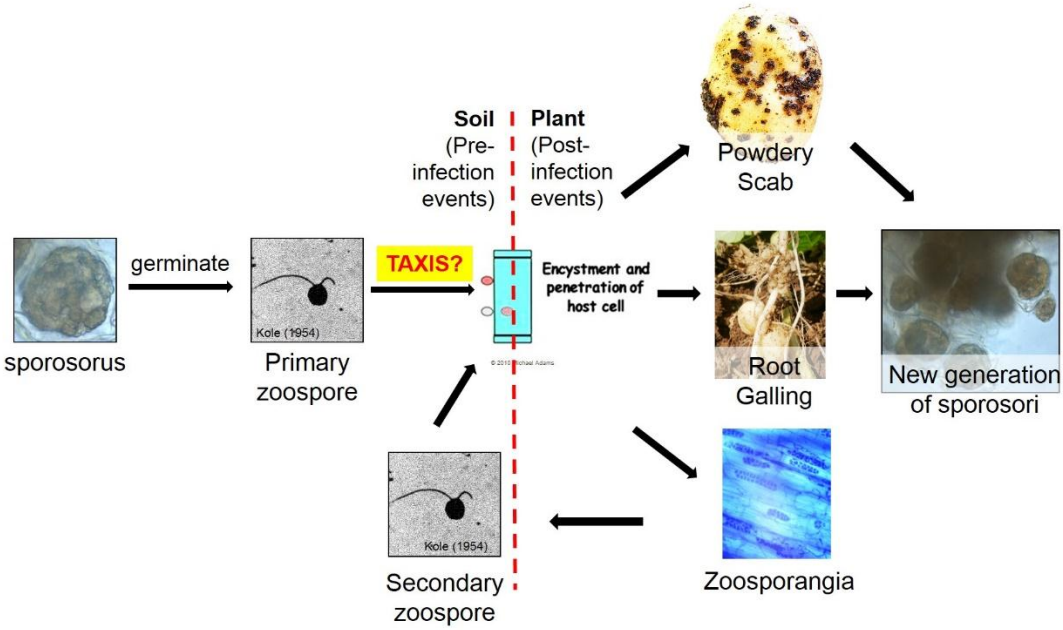

Figure 7. 3. The missing link. Are zoospores chemotactically attracted to chemical-compounds in root exudate and Hoagland's solution?

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Appendix A. Potato powdery scab: history and development (1841-1920)

A.1. In Germany: The First Report (1840's)

Potato arrived in Europe in the mid-1500's via Spain and England. Potato initialy did not receive acceptance due to its growth habit and cultivation requirements. The lack of proper cooking instruction was also believed to have contributed to the people's sceptism over potato as a food. However, King Frederich II of Prussia (1712-1786) realised how important potato could be in feeding the growing population in the rapidly booming industrial Germany. He introduced potato by tricking farmers. In 1740, he planted potato in Berlin with guards surrounding the field. The trick was to show farmers that potato is so important that the cultivation requires royal guards to keep out thieves. Not long after, the once curious and skeptic farmers began planting potato. Potato became a staple food in Germany. Frederich later received recognition and was named *Der Kartoffelkonig* or the "potato king". This was the beginning of potato cultivation in Germany (Agropa Handesl GmbH). Germany became one of the biggest producers of potato in Europe during that time. When King Frederich II died, people commemorated him by placing potatoes on his tombstone which still exist today (Christom Niemann, The New York Times). However, during the widespread potato cultivation, in the mid-9th century, potato diseases plagued Europe. The most devastating was the potato late blight (*Phytophthora infestans*), the culprit of the Irish Famine. There were also other diseases which were reported in that period that but did not receive the same attention as potato late blight. These diseases were causing losses, and worries, to farmers.

In February 1842, a German scientist by the name of R. W. Wallroth[3] scientifically described a potato disease which was characterised by pustules appearing on the surface of the tuber. The disease, which was known as "Knollenbrand" was already observed by farmers long before it was scientifically reported. Wallroth designated *Erysibe subterranea* as the causal organism, due to the pathogen's resemblance to smut. In the same year of the disease' first scientific report, the german botanist and explorer Karl Friedrich Philip von Martius illustrated microscopically (Figure 2) what Wallroth had observed. Martius, however, did not agree to naming the pathogen as *E. subterranea.* Instead, he described the causal organism as *Protomyces tuberum solani. Protomyces* species cause swelling of infected plant parts. The protruding pustules

[2] This manuscript is a work in progress

[3] According to W. J. Morse, Wallroth sent the report on September 22, 1841 and presented the paper in a meeting in Brunswick, Germany. However, the paper was published 5 months after the meeting (February 1842).

on the tuber surface may have led to the Martius decision to assign the genus *Protomyces* for the powdery scab pathogen. Martius illustrations were the subject of M. J. Berkeley's monograph in 1848. Berkeley, in London, commented on the tuber disease and was the first to indicate that the tuber disease observed by Wallroth and Martius was different from another scab disease (Berkeley was likely referring to common scab or wart). Our focus, however, is the tuber disease which Wallroth and Martius was referring to. The disease now known as "powdery scab".

A.2. From Erysibe to Spongospora subterranea (late 19th century)

When Berkeley studied powdery scab, he disgreed with both Wallroth's and Martius' taxonomic identity of its pathogen. Berkeley named the organism *Tuburcinia scabies*. Prior to Berkeley's identification, Rabenhorst in 1844 implied that Wallroth misidentified the pathogen. Rabenhorst re-described the pathogen as *Rhyzosporium solani*. Rabenhost work, however, did not receive recognition from the scientific community as evident in succeeding reports.

In 1856, E. Mercklin reported the same tuber disease in Moscow, Russia. Twenty-seven years later from Berkeley's study, the prominent Russian botanist Alexandr Alexandrovich Fischer von Waldheim changed the genus of the potato scab pathogen from *Tuburcina* sp. to *Sorosporium* sp., which appeared in his book "Apercu systematique des Ustulaginees" published in 1877. The Russian botanist agreed with Wallroth by placing the pathogen in the smut-causing group of pathogens. von Waldheim, in acknowledgement to Berkeley's contribution, retained the specific identity (*scabies*) of the powdery scab pathogen. When the botanist J. Brunchorst, in 1886 observed a similar potato scab symptom (locally known as "Skorv") in Norway, he conducted further studies. He concluded by naming the pathogen as *Spongospora scabies*.

Somewhere in Quito, Ecuador, on June 1892, the botanist and professor Gustaf de Lagerheim noticed black warts on potato tubers whilst collecting samples for bacterial cultures. He remarked that the disease is well known in Quito. Quito is the key political region of Ecuador, which is located in the Central Andean region. This region is known to be the center of origin of potato. He brought samples to the microbiological laboratory for microscopic examination. What he saw in the microscope resembled features reported by Brunchorst. However, de Lagerheim bluntly commented that Brunchorst made an erroneaous interpretation of what the Norweigan botanist saw microscopically. de Lagerheim wrote "the wart-forming tissue, which he [Brunchorst] considers as a part of the potato altered by the disease, is the pseudo-parenchyma of fungus hypha, in which the characteristic spore balls arise. The fungus is,

therefore, not a Myxomycete, and has no relation to Plasmodiophora." de Lagerheim observed warts which did not contain spores and were filled with protoplasmic membrane. He added "It is perhaps this vacuolated protoplasm which Brunchorst mistook for the plasmodia of his *Spongospora*." de Lagerheim, of course, was proven incorrect by the succeeding studies in the dawn of the 20th century. Despite this, de Lagerheim's contribution remains evident in the current time, *Spongospora subterranea* (Wallroth) Lagerheim – the current taxonomic name of the powdery scab pathogen. Some of the early 20th century papers, however, did not acknowledge de Lagerheim's contribution and instead have credited professor Thomas Johnson.

A.3. From British Isles to North America (early 20th century)

Thomas Johnson, a british paleobotanist who later served as a professor of botany at the Royal College of Science, in Dublin[4], presented and read several papers on potato tuber diseases prevalent in Ireland during the scientific meetings of the Royal Dublin Society. From 1907 to 1909, his studies included *Spongospora solani* (Brunch.). While visiting the Dingle peninsula, Johnson observed scabbiness of tubers and upon close examination, saw spore balls of *S. solani.* The Dingle peninsula is among the many regions in Ireland that was hit by the potato famine in 1845. Johnson talked with the farmers and asked why powdery scab was rampant in the region. Many farmers planted continuously the already infected tubers because they thought that scabbines was an indication of tuber excellence and superiority. Johnson described and studied the powdery scab pathogen in details for the first time. He was responsible for associating "corky scab" to the tuber disease. Johnson established the first official seed-testing stations in Ireland and Britain[5]. However, Johnson made no quote of *Spongospora subterranea* and did not mention de Lagerheim's 1982 observations. Johnson unified Berkleys *S. scabies* and Brunchost *S. solani.* Johnson also reassigned the powdery scab pathogen from the smut group back to the myxomycete group. Johnson saw no difference on the structure of the organisms based on his comparison of herbarium samples which he received from Colonel Prain, Director of the Royal Botanic Gardens in Kew. Johnson retained *Spongospora solani* (Brunch.) as the causal pathogen and a pathogen which had properties of the pathogens belonging to the myxomycete group. The american plant pathologist Irvin Melhus commented that Johnson should be credited for the etymology (origin and change of the name) of the powdery scab pathogen.

[4] Stafleu F. A. and Cowan, R. S. 1976. Taxonomic literature: a selective guide to botanical publications and collections with dates, commentaries and types. Volume 1 A-G.
[5] University College Dublin. From early scientific to today's UCD Science - Towards a history of UCD College of Science. Page 11.

Appendix B. Tomato root infection by *Spongospora subterranea* results to poor plant growth

B.1. Abstract

The susceptibility of three tomato cultivars to *Spongospora subterranea* and the effect of *S. subterranea* root infection to tomato growth at seedling stage were investigated. Tomato cvs. Grape, Truss and Roma were grown and challenged with *S. subterranea* in a hydroponic system. In three glasshouse trials, important plant growth traits of tomato cv. Truss were assessed by comparing seedling stage functional traits between plants grown in non-inoculated and *S. subterranea*-inoculated hydroponic solution. Moderate to severe zoosporangia infection was observed, but no significant variation in susceptibility was recorded among the three tomato cultivars ($P=0.344$). Plants grown in *S. subterranea*-inoculated solution had shorter ($P<0.05$) plant length, lighter ($P<0.05$) plant weight, smaller ($P<0.05$) leaf surface area and were less vigorous ($P<0.05$) compared to plants grown in non-inoculated solution. The findings from this study indicate that tomato cultivar differences do not affect *S. subterranea* disease severity and demonstrate that *S. subterranea* root disease results to poor plant growth and development. The latter findings may have implications in yield response of crops that are host of *S. subterranea* and may help explain the contrasting reports on yield effect of *S. subterranea* root infection to its other host-plant, potato.

Keywords: powdery scab, zoosporangia, plasmodiophorid, tomato-bait assay

B.2. Introduction

Spongospora subterranea f. sp. *subterranea* is a soil-borne pathogen and is the causal agent of powdery scab in potato tubers (Wallroth 1842). The pathogen is an obligate biotroph relying solely on living host plant to complete its life cycle and thus they are unable to growth in synthetic media (Braselton 1992). Pivotal to the disease development process is the germination of resting spores (survival and protective structures) resulting to the release of short-lived primary zoospores (the infective agent) (Chapter 2A). Infection of *S. subterranea* in a susceptible host plant can occur as

early as seedling stage up to plant maturity and early host-pathogen interaction can lead to greater disease development (Thangavel et al. 2015). Within the roots, zoosporangia are formed and secondary zoospores are produced (Kole and Gielink, 1963). This polycyclic event can occur several times, re-infecting the roots, and thus there is an abundance of active inoculum that are ready to infect at potato tuber initiation (Merz and Falloon, 2009). In potato, root galls are formed at some stage during zoospore infection. Galling occurs because of resting spores proliferating in potato tissues. The impact of *S. subterranea* root infection in potato field have been assessed (Johnson and Cummings, 2015, Falloon et al. 2016), but it was not clear whether the observed effect was the result of zoosporangia infection or by root galling.

While *S. subterranea* is commonly studied in potato, several alternative host of *S. subterranea* have been reported (Chapter 2B). However, most of these plants produce only zoosporangia and quite a few that forms into root galls. There are 28 plants species that have been associated with *S. subterranea* root infection and several of them belong to the Solanaceae family. The most common is tomato as it is widely used as bait-plant for biological assays (Merz, 1989). Reports from Europe and Oceania indicate that only zoosporangia are formed in tomato, but the Americas indicate otherwise (Chapter 2B). In Australia, infection of *S. subterranea* in tomato cv. Roma resulted to severe zoosporangia formation and no root galling (de Boer and Theodore, 2000). Using tomato cultivars that supress root galling may help determine which stage of *S. subterranea* root infection has detrimental effect to host-plant health.

Using tomato, the plant-bait test or bioassay allows rapid detection of *Spongospora subterranea* in the soil (Flett, 1983) or in a solution (Kole, 1954, Merz, 1989). This approach is particularly useful in studying the viability and infectivity of resting spores. Critical to the bioassay is the susceptibility of the bait plant, tomato. All cultivars used in previous studies were susceptible, but no standard tomato cultivar had been established. For instance, cv. Montfavet H 36-5 had been used in Switzerland (Merz, 1989), cv. Moneymaker in Scotland (Stuart Wale, *personal communication*) and cv. Roma in Australia (De Boer, 2000). Furthermore, bioassays or infectivity tests have been also performed under different conditions with different sources of resting spore and varying loads of inoculum. Thus, variations in severity of tomato root infection may have been caused by cultivar differences or by disparity in bioassay conditions. The former could affect the assessment of plant and yield impact of root infection, and the latter may influence cultivar resistance screening.

This study examined the susceptibility of three tomato cultivars to *S. subterranea* and investigated the impact of zoosporangia root infection on tomato-plant functional traits.

B.3. Materials and Methods

B.3.1. Assay 1

Powdery scab-infected potato tubers, collected from Devonport, Tasmania, were washed for 1-2 minutes with running tap water, soaked for 3 minutes in 2% bleach solution, rinsed quickly and air-dried. Scab lesions were removed using scalpel and excised lesion was placed in McCartney bottle and oven-dried for 4 days at 40°C. The dried tissues were ground using mortar and pestle and the "tuber" inoculum was quantified in a haemocytometer under a light microscope (DM 2500 LED, Leica Microsystem, Germany). The inoculum contained 6,900 sporosori/mg. Tomato seeds of cultivars Grape, Truss and Roma were extracted from the fruits obtained from a commercial supermarket (Coles Supermarkets Australia Pty Ltd, New Town, Tasmania). Seeds were air-dried for 3–5 days at room temperature, and placed in a McCartney bottle with cotton plugs (to absorb moisture) and stored at 4°C until use. Seeds were sown in a nursery grade coco peat-based potting mix (Horticulture and Landscape Supplies, Tasmania) and grown in the glasshouse for three weeks. The germination rate was 80% in Grape and Truss, and 50% in Roma. Three-week-old healthy plants of all cultivars were uprooted and roots washed to remove adhering soil. Fifteen plants (5 replicates of each cultivar) were placed in a modified rectangular container (size, and spacing between plants) containing a ¼ strength Hoagland's solution (Shah *et al.*, 2012) and 20 mg of pre-conditioned inoculum (Merz 1997). Plants were grown in the glasshouse at 22 ± 2°C in a Completely Randomized Design (CRD) with five replicates. After three weeks' incubation, plants were removed from the solution, assessed for root infection and plant functional traits measured. Tomato cultivars grown in Hoagland's solution without *S. subterranea* resting spores served as the non-inoculated control check.

B.3.2. Assay 2

In this assay, the effect of a *S. subterranea* gall inoculum designated as "gall clean", which was prepared to reduce the number of inoculum-borne microbes, on root infection was examined.

Because *S. subterranea* is non-culturable, tuber or gall inoculum used in pathogenicity studies had considerable number of other microbes. Some strategies have been developed to study *S. subterranea* inoculum in isolation. Qu and Christ (2007) maintained *S. subterranea* in potato root hairs. An improved approach used by Bulman *et al.* (2011) reported that root galls, containing *S. subterranea* resting spores, can be maintained in a culture media, without apparent contamination. This approach is promising because it allowed the production of relatively cleaner inoculum compared to those extracted directly from root galls and tuber scabs. Also, because galls were mounted on a culture media, microbial contamination was easily monitored. In this study, "gall clean" inoculum was prepared following a modified extraction method (Figure B.1) similar to that described by Bulman *et al.* (2011). Essentially young white root galls were collected, washed in running water for 30 min, soaked in 2% bleach solution for 2.5 min, washed thrice in sterile distilled water and air-dried on sterile tissue paper. The galls were than mounted on callus inducing media (MS salts, 4.43 g^{-1}; sucrose, 30 g^{-1}; NAA, 1 mg^{-1}; Kinetin 1 mg^{-1}; Type A Agar, 8 g^{-1}; pH, 5.8) until use. All chemicals used in this study were sourced from Sigma (USA), unless otherwise stated. Since no significant variation in root infection was observed among the three cultivars, only cv. Grape was assayed. Tomato cv. Grape was chosen because of its percent germination rate and availability of seedling materials. Healthy tomato cv. Grape plants were individually placed into McCartney bottles containing 20 ml of distilled water and inoculated either with five "gall clean" inoculum. Plants were grown at 15-18°C, exposed to 16 hours' light (24 hours cycle) and 60% humidity in a growth chamber. After three weeks from incubation, plants were removed from the solution, assessed for root infection and plant functional traits measured. Plants in distilled water containing no-resting spore inoculum served as control check.

B.3.3. Assay 3

In this assay, the effect of three inoculum source ("tuber", "root gall" and "clean gall") inoculum on root infection and plant functional traits was examined, although the inoculum concentration was not measured. All resting spore-inoculum source was collected from the same plant source. The "root gall" inoculum was processed following the procedure used in the "tuber" inoculum. Healthy tomato cv. Grape plants were individually placed into McCartney bottles containing 20 ml of distilled water and inoculated either with 1 mg of "tuber", "root gall" and five young "clean gall" inoculum. Bioassay condition was identical to assay 2. After two and six weeks

from incubation, plants were removed from the solution, assessed for root infection and plant functional traits measured.

B.3.4. Root "Zoosporangia" Infection and Plant Functional Traits Assessment

Roots were washed, cut and placed on a microscope slide. Root samples were stained with 0.1% trypan blue for at least 10 minutes. Formation of zoosporangia (Ledingham, 1935) was assessed under light microscopy at 200X magnification, photomicrographs were taken at 400X magnification. Severity of zoosporangia were assessed using the severity rating scale (Merz *et al.*, 2004): 0 (no infection), 1 (sporadic, zoosporangia covering approximately 1% of the roots), 2 (slight 2-10%), 3 (moderate, 11-25%), 4 (heavy, 26-50%) and 5 (very heavy, >50%). Root browning (0-5, 0-no browning, 5-extreme browning) was also recorded. Important plant functional traits (Cornelissen *et al.*, 2003) were measured. These are root and shoot length or plant height (cm), root and shoot fresh weight or plant biomass (mg), leaf surface area or leaf size (cm^2) and relative plant vigour (1-10, 1-less vigorous, 10-most vigorous). Relative plant vigour was recorded by selecting the most vigorous plant (score of 10) then comparing it to all other plants in all treatments. For leaf surface area, aerial photographs (including a pen to scale the length) of leaves were taken and leaf surface area was then calculated using the ImageJ 1.x software (Schneider et al 2012).

B.3.5. Data Analysis

A one-way analysis of variance (ANOVA) was performed to determine the statistical significance between means of three treatments at 0.05 level. The effect of root infection on various plant functional traits was analysed by comparing the treatment means from *S. subterranea*-inoculated and non-inoculated treatments. Comparison of treatment means was performed using an independent Student's t-test analysis at 0.05 level of significance, except in the root infection development (comparison of data between 3 and 6 weeks' incubation) where a dependent t-test analysis was performed. All analyses were carried out using a SPSS® statistical software (Version 22, Armonk NY, USA).

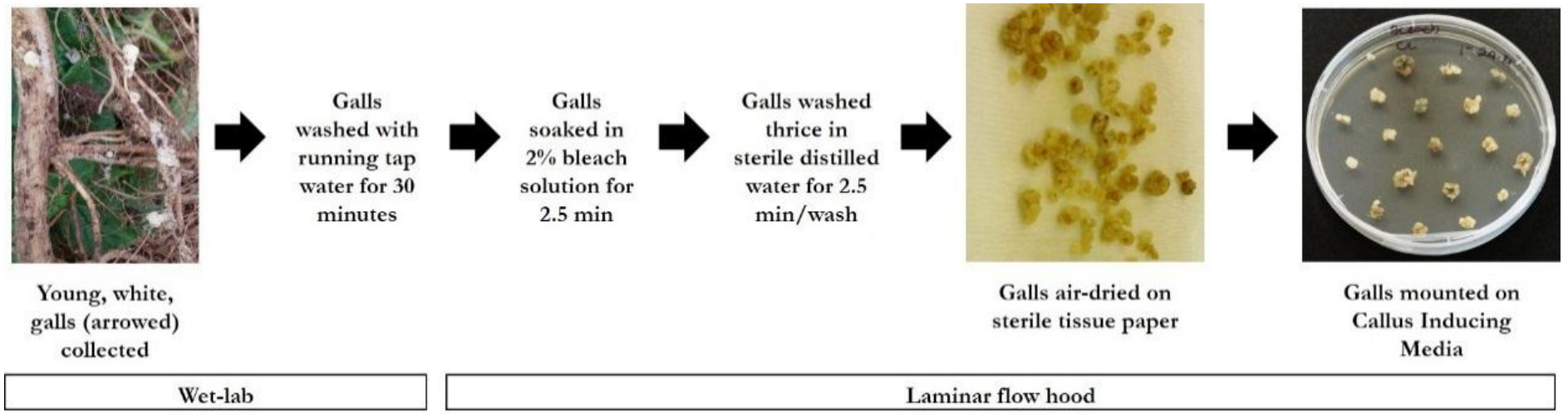

Figure B.1. Schematic flow of root gall clean-up procedure used in the study, based on the procedure used by Bulman *et al.* (2011).

B.4. Results

B.4.1. Assay 1

All cultivars were very susceptible to *S. subterranea* with the highest zoosporangia (Figure B.2.A and B.2.B) score recorded in Grape (4.2), followed by Roma (3.8) and then Truss (3.6). However, the mean difference in the severity of zoosporangia among the three cultivars was not significantly different (*P=0.344)*. Zoosporangia infection had significant detrimental effects on plant health. Significant reduction (*P<0.05*) in root length, shoot length, leaf surface area (Figure B.2. C) and plant vigour (Figure B.2. D) were observed (Figure B.3).

B.4.2. Assay 2

The "gall clean" inoculum showed visually less contamination than the non-cleaned gall (Figure B.4). In the absence of other microbes, mean zoosporangia were moderately severe (Table B.1). Root infection also had significant (*P<0.05*) negative impact on the tomato growth parameters. *Spongospora subterranea*-infected plants were 65% lighter (plant biomass), 83% narrower (leaf surface area) and 30% shorter (plant height) than the non-infected plants. While roots of *S. subterranea*-infected plants was 37% shorter than the roots of non-infected plants, the difference was not significant (*P*=0.071).

B.4.3. Assay 3

A similar trend was observed with that in assays 1 and 2. Zoosporangia infection lead to a reduction in tomato growth, regardless of the inoculum source at two (Figure B.5) and six (Figure B.6) weeks after incubation assessment periods. Development of shoots traits and plant vigour were negatively influenced by *S. subterranea*, except in root traits using "tuber" inoculum (Figure B.7). Browning of roots was more severe in plants inoculated with the tuber inoculum and less in the "gall clean" inoculum (Table B.2). The non-infected plants were also more vigorous than the *S. subterranea* infected plants (Figure B.7, Table B.2).

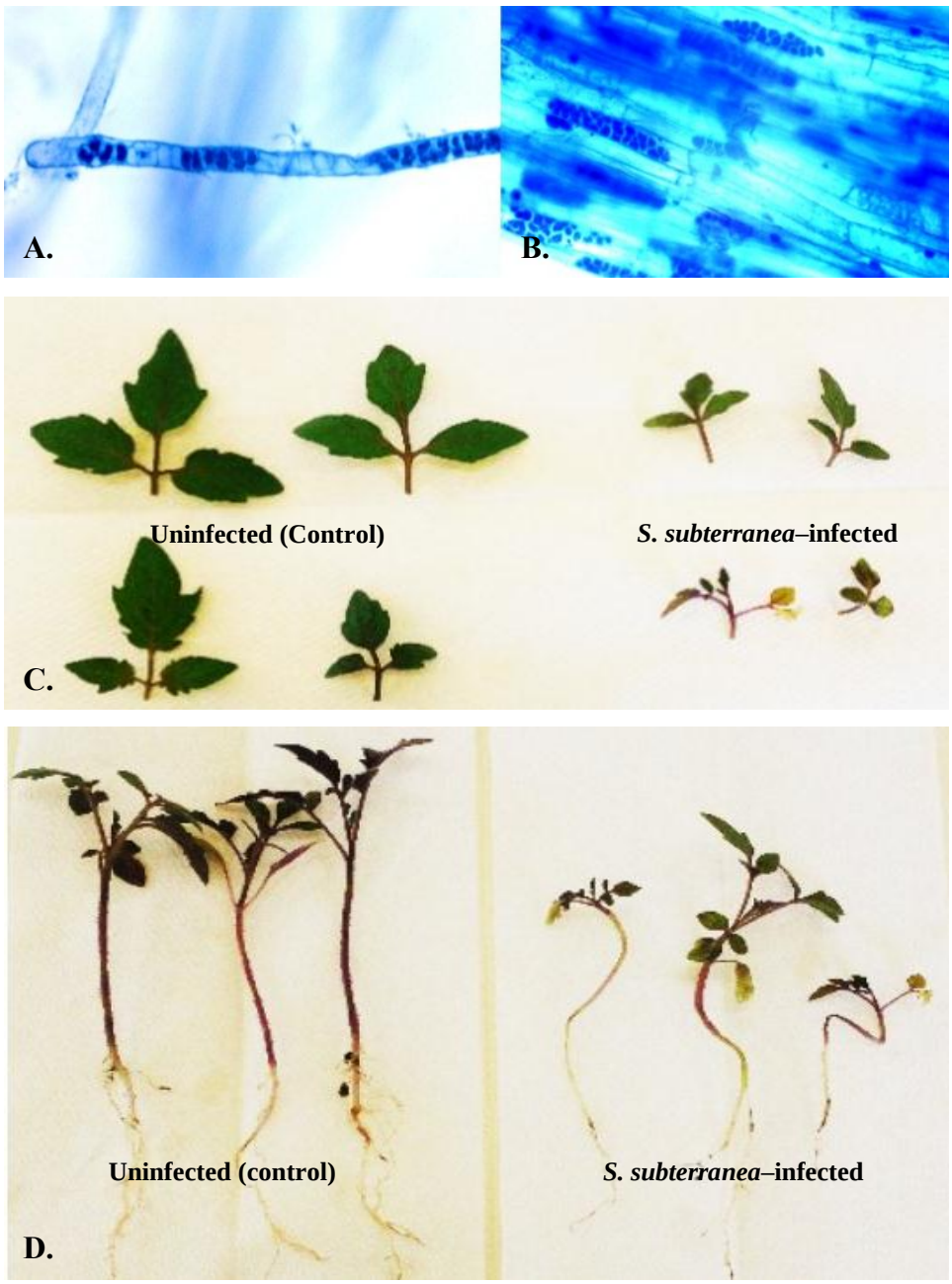

Figure B.2. *Spongospora subterranea* zoosporangia in tomato root hairs (A) and main root (B). Leaf surface area (C) and relative plant vigour (D) of uninfected and *S. subterranea*-infected tomato plants. Roots were stained in 0.1% trypan blue stain solution and plants were grown in hydroponics system.

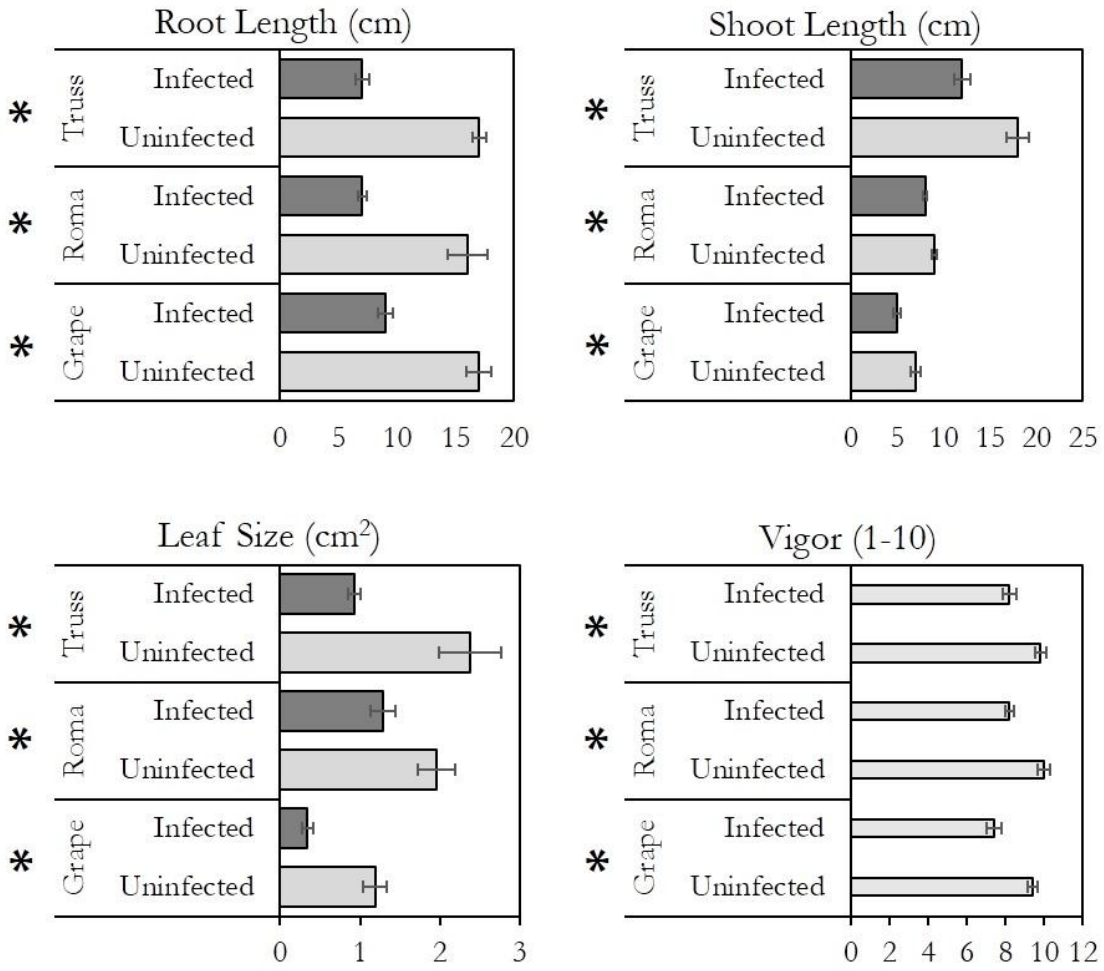

Figure B.3. Effect of *Spongospora subterranea* zoosporangia infection plant growth of three tomato cultivars. Horizontal bars are standard error (n=5). Asterisk indicate means are significantly different at 0.05 level of significance by independent Student's t-test.

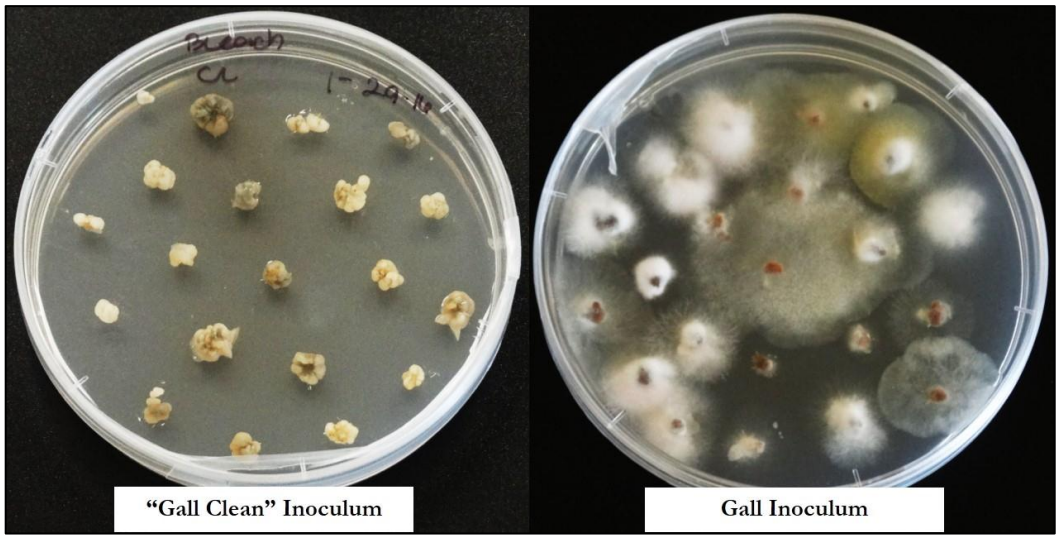

Figure B.4. Absence of other microbes in "gall clean" inoculum and microbial growth in non-cleaned gall inoculum. In "gall clean", white root galls were collected, washed in running water for 30 min, soaked in 2% bleach solution for 2.5 min, washed thrice in sterile distilled water and air-dried on sterile tissue paper. The galls were than mounted on callus inducing media (see materials and methods for components).

Table B.1. Severity of *Spongospora subterranea* zoosporangia infection and the effect of zoosporangia on 'Grape' tomato plant biomass, root length, leaf surface area and plant height.

	Non-infected	*S. subterranea*-infected	Percent Reduction	*t-test*
Zoosporangia (score)	0.00 ± 0.00	3.33 ± 0.33	-	*P*=0.001*
Plant Biomass (mg)	633.33 ± 28.48	223.33 ± 94.04	65 % ↓	*P*=0.014*
Root Length (cm)	7.97 ± 0.53	4.99 ± 1.09	37 % ↓	*P*=0.071
Leaf Surface Area (cm^2)	6.69 ± 0.90	1.13 ± 0.16	83 % ↓	*P*=0.004*
Plant height (cm)	16.52 ± 0.64	11.59 ± 1.15	30 % ↓	*P*=0.020*

Asterisk indicate means were significantly different at 0.05 level of significance by independent Student's t-test.

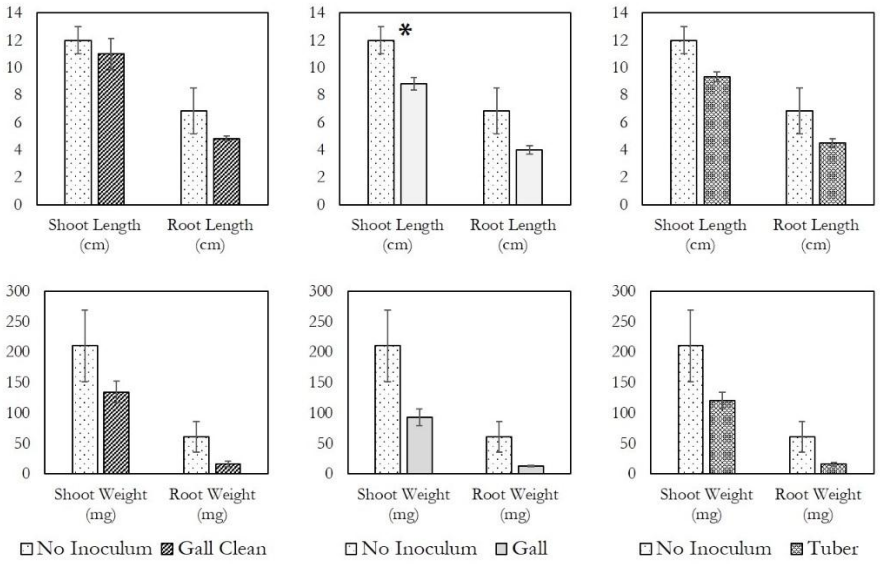

Figure B.5. Effect of *Spongospora subterranea* zoosporangia on 'Grape' tomato seedlings shoot and root length (upper) and shoot and root weight (lower) at two weeks after inoculation using "clean gall", "root gall" and "tuber" inoculum. Vertical bars are standard error (n=3). Asterisk indicate means are significantly different at 0.05 level of significance (Student's t-test).

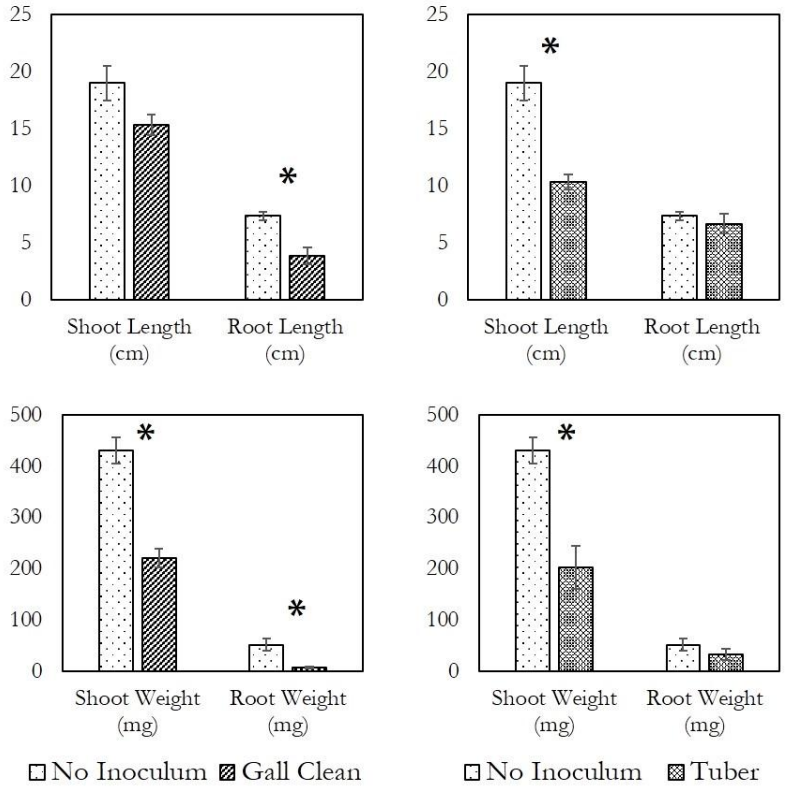

Figure B.6. Effect of *Spongospora subterranea* zoosporangia on 'Grape' tomato seedlings shoot and root length (upper) and shoot and root weight (lower) at two weeks after inoculation using "clean gall" and "tuber" inoculum. Vertical bars are standard error (n=3). Asterisk indicate means are significantly different at 0.05 level of significance (Student's t-test).

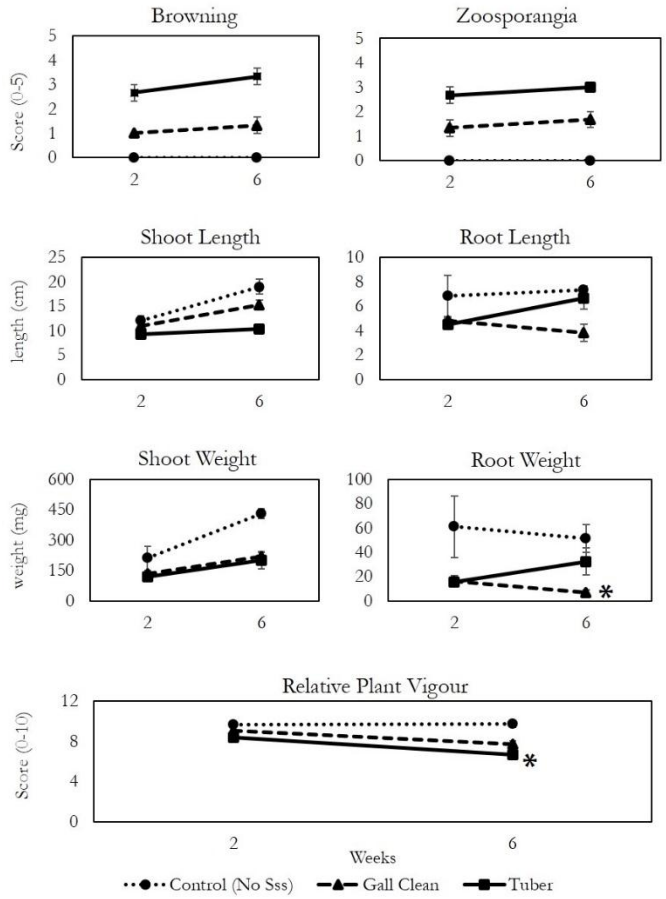

Figure B.7. Tomato cv. Grape seedling growth and infection parameters from two to six weeks from incubation with "gall clean" and "tuber" inoculum. Vertical bars are standard error (n=3). Asterisk indicate means are significantly different at 0.05 level of significance (dependent Student's t-test).

Table B.2. Effect of *Spongospora subterranea* inoculum source on the severity of zoosporangia, root browning and plant vigour of tomato.

Plant Age	Treatment	Zoosporangia Score (0-5)	Root Browning (0-5)	Plant Vigour (0-10)
2 weeks	No Inoculum	0.00 (±0.00) [a]	0.00 (±0.00) [a]	9.66 (±0.33) [a]
	Gall Clean	1.33 (±0.33) [b]	1.00 (±0.00) [b]	9.00 (±0.00) [b]
	Gall	2.66 (±0.33) [b]	1.66 (±0.33) [b]	7.33 (±0.33) [b]
	Tuber	2.66 (±0.33) [b]	2.66 (±0.33) [b]	8.33 (±0.33) [b]
6 weeks	No Inoculum	0.00 (±0.00) [a]	0.00 (±0.00) [a]	9.67 (±0.33) [a]
	Gall Clean	1.67 (±0.33) [b]	1.33 (±0.33) [b]	7.67 (±0.33) [b]
	Tuber	3.00 (±0.00) [b]	3.33 (±0.33) [b]	6.67 (±0.33) [b]

Means of "gall clean", "root gall" and "tuber" inoculum treatments were significantly different to the non-inoculum treatment at 0.05 level of significance (independent Student's t-test).

B.5. Discussion

Tomato cvs. Grape, Truss and Roma were highly susceptible to *S. subterranea* and infection resulted to severe zoosporangia formation. *Spongospora subterranea*-infected tomato plants were generally shorter, lighter, narrower in leaf surface area and less vigorous compared to the uninfected plants. Similar pattern of negative effects was recorded when "tuber", "root gall" and "clean gall" inoculum sources were independently used.

Tomato is susceptible to *S. subterranea*, which makes it an ideal model bait-plant for zoosporangia bioassays (Merz, 1989). However, selection of resistance and susceptible tomato cultivars to *S. subterranea* root infection received little attention since all tomato cultivars assayed worldwide showed susceptibility to the pathogen. In this study, we observed that root infection by *S. subterranea* in three tomato cultivars, grown in a hydroponic system and receiving the same bioassay condition, resulted to significantly comparable disease severity. The findings indicate that although there were differences in the severity of tomato root infection noted in previous studies (Kole 1954, Merz 1989, De Boer 2000), the severity of infection was less likely caused by the variations in tomato cultivars that were used as bait-plants. Our study confirms cv. Roma as a very susceptible host of *S. subterranea* in the soil-less assay, complementing the work of De Boer (2000) on soil. This is the first report of *S. subterranea* infection in tomato cvs. Truss and Grape, making these two cultivars as suitable alternative host-bait plants for zoosporangia bioassays.

Tomato plant functional traits was negatively influenced by the presence of *S. subterranea* zoosporangia. Ledingham (1935) reported that root infection by *S. subterranea* leads to zoosporangia formation. Kole and Gielink (1963) elucidated that zoosporangia are the source of secondary zoospores. This study demonstrates that zoosporangia are not only the source of secondary zoospores, but its presence in the host roots also has potential impact on tomato plant's health. The poor growth and development of *S. subterranea*-infected plants could have been due to poor water and nutrient uptake by plants (Falloon et al 2004, Lister et al. 2004). Recently, Falloon *et al.* (2016) reported that root diseases caused by *S. subterranea* negatively affects potato plant productivity. However, there was no reference of the root disease component (zoosporangia or root galling) which caused the reduced yield productivity. There is an indication, observed by Johnson and Cummings (2015), that root galling is less likely causing potato plant yield loss. Although the effect of soil on plant growth and development

and potential variations between tomato and potato have not been considered, the findings from this study clearly showed that early and severe zoosporangia root infection alone resulted in poor plant growth and delayed the plant's development. The poor tomato plant functional traits recorded in this study also complimented the observations of Falloon *et al.* (2016) in potato. This study, therefore, demonstrates that reduced potato productivity observed in the field is more likely the result of severe zoosporangia infection and less-likely by root galling. This study could potentially explain the contrasting results from the recent studies (Johnson & Cummings, 2015, Falloon *et al.*, 2016) on the impact of *S. subterranea* root infection on potato yield. In potato breeding perspective, our findings suggest that among the three *Spongospora* diseases components (Wallroth, 1892, Ledingham, 1935, Pethybridge, 1912 , Falloon et al 2016), a great deal of importance should be given to zoosporangia resistance screening if the main objective is to avoid plant yield loss. However, there is no clear link between root and tuber disease resistance. Hence, if zoosporangia resistant line is selected there is a chance that the line is susceptible to powdery scab, which is the case of several potato cultivars. This is challenging because tuber quality/marketability is paramount to a profitable potato farming. Thus, breeding programs may have to depend on the already known potato cultivars/lines with relative resistance to tuber powdery scab and focus on improving root disease resistance to these cultivars/lines using the available biotechnology tools and techniques, e.g. inducing plant resistance by chemicals (Hernandez-Maldonado et al. 2014), somaclonal cell selection (Wilson et al, 2010).

Using a refinement of Bulman *et al.* (2011) procedure, we were able to obtain a relatively cleaner collection of *S. subterranea* inoculum. Browning was reduced in plants that received the "gall clean" inoculum compared to plants that received the un-cleaned "tuber" and "root gall" inoculum. Browning is commonly associated with the presence of saprophytic or rot causing microbes (Harrison et al. 1997). Some of these microbes can be pathogenic (Dorojkin, 1936, Foister *et al.*, 1952, Diriwachter & Parbery, 1991) and hence, when an inoculum is used assessing the effect on plant health, there can be doubts whether the observed effect is being caused by the pathogen of interest, *S. subterranea,* or by other soil-borne microbes. No correlation analysis was performed due to the limited number of sample observations, but there is a trend of reduced zoosporangia and reduced browning, which also suggests that presence of other soil microbes may have exacerbated the negative effect observed using "tuber" and "root gall" inoculum on plant functional traits. While it cannot be determined if this exacerbated negative effect was caused by variations in inoculum concentration or presence of other inoculum-borne microbes,

using an inoculum, which had considerably less contaminating soil-borne microbes, still resulted to severe zoosporangia infection leading to poor plant growth and development.

In conclusion, difference in tomato cultivars does not influence *S. subterranea* zoosporangia severity, but early and severe zoosporangia infection can lead to poor tomato plant growth and development.

Appendix C. Inhibition of zoospore encystment is involved during the expression of potato resistance to *Spongospora subterranea*

C.1. Abstract

Biotrophic plant pathogens feed and extract nutrients from a living host. Infection by biotrophs can be primarily avoided by preventing the pathogen from entering the host. *Spongospora subterranea* is a biotrophic plasmodiophorid species that causes powdery scab and root infection in potato. Several potato cultivars have shown resistance to *S. subterranea*. To understand the mechanism of plant resistance, this study investigates if *S. subterranea* encystment is prevented in the resistant cultivars. In an *in vitro* bioassay aided by light microscopy, detached-roots from various potato cultivars were exposed to *S. subterranea* zoospore suspension for a short-period. The cultivar's disease resistance was correlated significantly with the number of zoospores encysted in the roots. The number of zoospores encysted decreased as host resistance to *S. subterranea* increased, and vice-versa. These findings indicate that resistance to *S. subterranea* may involve the host preventing zoospores from encysting. Further examination of cell-wall structures, and the associated genes, of susceptible and resistant plants may help further elucidate the mechanisms responsible for inhibiting zoospore encystment.

Keywords: Powdery scab, *Spongospora* root infection, plasmodiophorid, potato disease, host-resistance, plant-pathogen interaction

C.2. Introduction

Biotrophic plant pathogens obtain nutrients from a living host (Gan *et al.*, 2012). When natural openings are non-accessible, biotrophs may penetrate the host tissue. Biotrophic pathogens must reside within the host to reproduce and complete its life cycle. Therefore, infection or disease development can be avoided if these pathogens fail to enter the host tissue. Resistance of plants to biotrophs can be expressed in several ways (Guest & Brown, 1997). Plants may release compounds that inhibits growth or germination of pathogen propagules. In the absence of these compounds, an intact and impenetrable barrier, e.g. cell wall, serves as the next defensive structure. However, when the barrier is breached, compounds released by the plants because of pathogen invasion may defend further pathogen invasion. This preventive

mechanism is initiated by the presence of toxic or inhibitory compounds. When biotrophs can establish infection within host tissue, resistant plants can delay or retard the development of the pathogen. These defence mechanisms against biotrophs are exhibited by plants expressing quantitative resistance.

Spongospora subterranea is a biotrophic plasmodiophorid pathogen that causes powdery scab and root infection in potato (Wallroth, 1842, Falloon *et al.*, 2016). The process of *S. subterranea* infection on its host (Chapter 2A) is likened to that of the process occurring in other plasmodiophorids (Keskin & Fuchs, 1969). Biflagellate zoospores (infective agents) are released from resting spores (survival structures). Zoospores then dock on the host surface. They penetrate the cell wall using a specialised structure (Rohr) and inject a substance (Stachel) into the host (Williams, 1970). The establishment of the pathogen within host tissue leads to plasmodia development and subsequently to zoosporangia and/or resting spores. Several potato cultivars have shown reported resistance to *S. subterranea* (Falloon et al., 2003). Hernandez Maldonado *et al.* (2012) indicated that resistance to *S. subterranea* is likely expressed during zoosporangia development. However, it is unknown if resistance is firstly expressed during the initial interaction between *S. subterranea* zoospores and the host plant.

The aim of this study is to determine whether resistant cultivars prevent encystment of *S. subterranea* zoospores. The test was performed by exposing detached-roots of various potato cultivars to *S. subterranea* zoospore suspension for a short-period and quantifying the number of encysted zoospores under light microscopy. The potato cultivars selected expressed varying resistance and susceptibility to *Spongospora* disease in the field (Falloon *et al.*, 2003). This study presents preliminary data suggesting that inhibition of zoospore encystment occurs in resistant cultivars. The findings of this study suggest that inhibition of zoospore encystment may precede the expression of further resistance during zoosporangia stage in resistant plants (Hernandez Maldonado *et al.*, 2012) and thus indicates that inhibition of zoospore encystment acts as the first line of host defence against *S. subterranea*.

C.3. Materials and Methods

C.31. Preparation of zoospore suspension

Zoospore suspension was prepared using the method described in Chapter 4. Briefly, sporosori (resting spore) inoculum was suspended in root exudate solution and incubated for at least 10 days at 15–18°C. The *S. subterranea* zoospore identity was verified by morphology (Kole,

1954) and motility behaviour (Merz, 1997) under a light microscope (DM 2500 LED, Leica Microsystem, Germany) at 200X magnification. Further verificatory test (Chapter 4) was performed by exposing whole roots of tomato cv. Grape plants to zoospore suspension for 24 hours then further incubation for at least 2 weeks. This bait-test (Merz, 1989) confirmed that zoospores formed zoosporangia in susceptible roots after infection (Ledingham, 1935).

C.3.2. Preparation of Test Potato-Plants

Tissue-cultured potato plants (cultivars Agria, Iwa, Shepody, Desire, Gladiator and Russet Burbank) were provided by the Molecular Biology and Tissue Culture Laboratory (NTRL, TIA-UTAS). Plants were grown for 2 weeks in potato multiplication (PM) medium (MS salts and vitamins, 30 g/litres of sucrose, 40 mg/litres of ascorbic acid, 500 mg/litre of casein hydrolysate and 0.8% agar with pH adjusted to 5.8) under 16 h photoperiod in white fluorescent lamps (65 μmol $m^{-2}s^{-1}$) at 24°C.

C.3.3. Zoospore-Root Encystment Assay

A single, 2-cm long, fine-root hair was excised and placed on a microscope slide and flooded with 70 μl of zoospore suspension. This set-up was then incubated at room temperature for 10 minutes. To ensure all roots received a similar time-exposure to zoospores, the test was performed one root/cultivar/replicate at a time. The number of zoospores attached on the roots were counted under a light microscope, at 200X magnification, by scanning the whole? sample root. There were three replicates per cultivar. The assay was performed twice.

C.3.1. Statistical Analysis

An analysis of variance (ANOVA) was performed to determine the variation between treatments. The Tukey's HSD test, at 0.05 level of significance, was used for multiple comparison of means. A Spearman's correlation (r_s) test was then performed to determine the relationship between the number of zoospores encysted in various potato cultivars and resistance ratings of these cultivars in the field (Falloon *et al.*, 2003). The strength of the correlation was determined using the following guide; 0.00 – 0.19 = very weak, 0.20 – 0.39 = weak, 0.40 – 0.59 = moderate, 0.60 – 0.79 = strong, 0.80 – 1.00 = very strong. All statistical procedures were performed using the SPSS® statistical software (Version 22, Armonk NY).

C.4. Results

Spongospora subterranea zoospores encysted in all potato cultivars roots (Figure C.1). However, the number of encysted zoospores decreased as the rating of potato cultivars to *Spongospora* disease resistance increased (Table C.1). There was a strong, negative correlation between number of encysted zoospores and increasing resistance to *Spongospora* disease (Figure C.2). The inhibition of zoospore encystment by resistant cultivars Gladiator and RBK, and the negative relationship (zoospore number-resistance rating) were consistent in both Assay 1 (r_s=-0.782, df=13, p<0.01) and Assay 2 (r_s=-0.653, df=16, p<0.01).

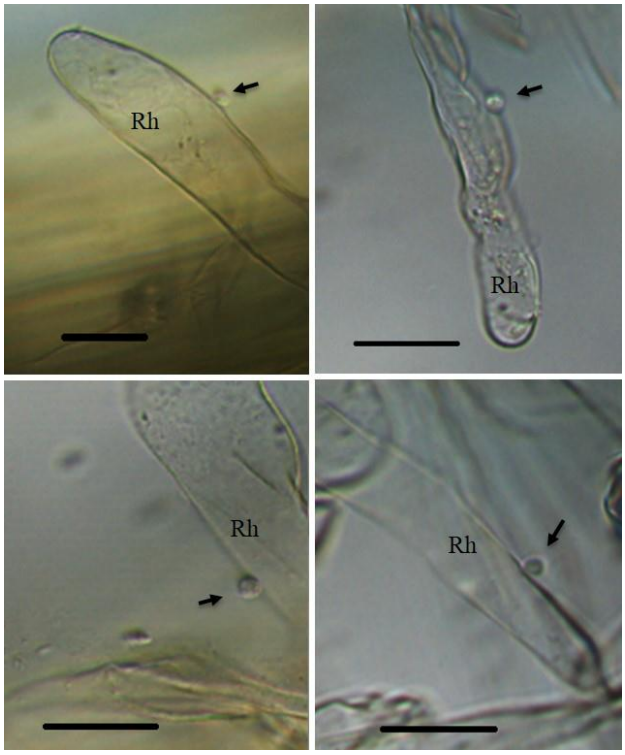

Figure C.1. *Spongosproa subterranea* zoospores (arrowed) encystment in potato root hairs (Rh). Bar = 30 μm

Table C.1. Effect of host cultivars on *Spongospora subterranea* zoospore encystment on detached roots.

Potato Cultivars	Reaction to PS		Assay 1		Assay 2	
	Falloon *et al.* (2003) [1]	Merz *et al.* (2012) [2]	Rank [3]	Mean zoospore count [4]	Rank	Mean zoospore count
Gladiator	Very Resistant	Resistant	5	3.70 ± 0.3 [a]	6	1.70 ± 0.9 [a]
Russet Burbank	Moderately Resistant	Resistant	4	3.00 ± 1.1 [a]	5	1.70 ± 1.2 [a]
Agria	Very Susceptible	Susceptible	2	7.70 ± 0.9 [ab]	2	10.3 ± 3.3 [ab]
Desiree	Moderately Resistant	Intermediate	3	13.3 ± 1.5 [bc]	4	13.7 ± 2.0 [bc]
Iwa	Very Susceptible	-	1	16.7 ± 2.6 [c]	1	16.0 ± 1.7 [bc]
Shepody	Moderately Susceptible	-		Ni [5]	3	20.3 ± 2.0 [c]
P value				*<0.01**		*<0.01**

[1] Based on field screening in New Zealand. Gladiator and Iwa were used as resistant and susceptible standards, respectively.

[2] Based on field screening in Europe.

[3] Rank of 1= very susceptible to 5 (assay 1) or 6 (Assay 2) = very resistant.

[4] Mean number of zoospores attached in the roots. Assay 1 and 2 were performed on May and August 2015, respectively.

[5] Not included

Values after ± are standard error (n=3). Means followed by the same letter within a column are not significantly different at the .05 level, using Tukey's HSD (n=3).

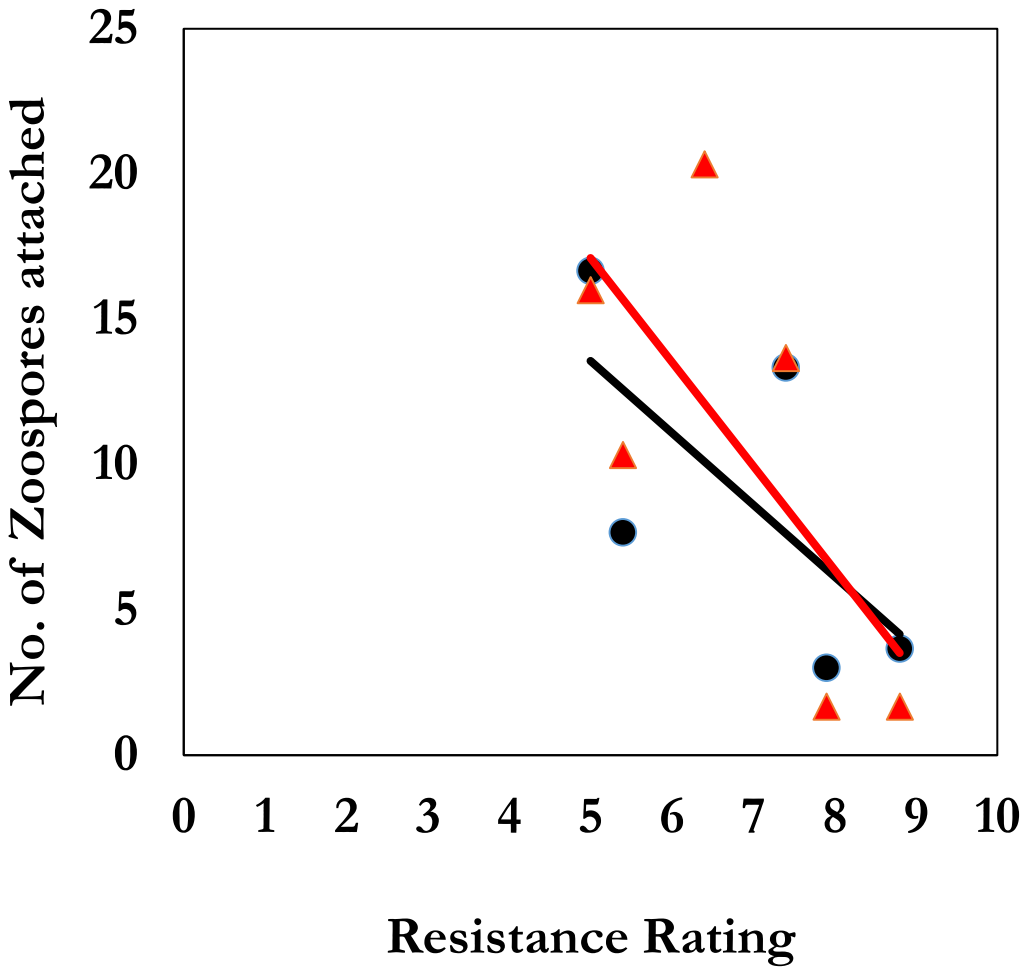

Figure C.2. Negative relationship between zoospore population settlement and increasing resistance to powdery scab. Assays 1 (●) and 2 (▲). Resistance rating, 1 = very susceptible, 10 = very resistant (Falloon *et al.* 2003). Spearman's correlation coefficient (r_s) in both assays are significant at the 0.05 level. Could r levels be placed on graph?

C.5. Discussion

Several potato cultivars have been shown to express resistance to *Spongospora* disease (Falloon et al., 2003). Here we found that the number of zoospores encysted on roots decreased when the resistance rating of potato cultivars increased. Conversely, this observation was reversed (zoospore encystment increased) when the potato cultivars susceptibility to *Spongospora* disease increased.

Studies strongly indicate that the germination of *S. subterranea* resting spores is stimulated by potato root exudates (Fornier *et al.* 1996, Chapter 4) and some compounds released in the root exudates (Chapter 4). Hernandez Maldonado *et al.* (2012) indicated that resistance in potato cultivars to *S. subterranea* is likely expressed in the zoosporangia stage, within the plant tissue. Since zoospore encystment precedes zoosporangia development, this stage is likely the first line of defence expressed by resistant potato cultivars against *S. subterranea* invasion. Similarly, this is also the first line of defence in resistant-host plants to *Plasmodiophora brassicae,* also a biotrophic pathogen within the same group as *S. subterranea* (Braselton, 2001). This reaction was also associated with the production of α 1-3 glucan (callose) that is rapidly deposited in pathogen-infected cells (Donald & Porter, 2014). Further investigation of the role of α 1-3 glucan may shed light into the anatomy of potato resistance to *S. subterranea* invasion and further work in this aspect is needed.

Quantitative resistance of plants to the pathogen may reduce the chance of invasion or retard the development of infection (Guest & Brown, 1997). The findings of this study support previous work indicating that resistance to *S. subterranea* is quantitative (Falloon, 2008). The variations expressed by different potato cultivars during zoospore encystment indicated partial resistance to *S. subterranea*, as no cultivar completely inhibited zoospore encystment. Since *S. subterranea* life cycle is polycyclic (enters a secondary infection phase) (Kole & Gielink, 1963) but fewer zoospores encysted in resistant plants, the build-up of epidemic will be lower compared to those in susceptible plants (Van Der Plank, 1963). This build-up of epidemic is also likely delayed during the zoosporangia stage of infection within the root tissue (Hernandez Maldonado *et al.*, 2012). Furthermore, since rating scores used in this study was based on the tuber infection severity (Falloon *et al.*, 2003) but response observed was correlated with zoospore encystment in the roots,

the findings of this study may suggest a link between tuber and root resistance to *S. subterranea* (Merz et al., 2012).

Of note, the cultivar Desiree behaved differently. Desiree is reportedly moderately resistant, although it has been shown as susceptible in other field studies, to powdery scab (Falloon *et al.*, 2003). However, the number of zoospores settled in Desiree were greater than the susceptible cultivar Agria. Merz *et al.* (2004) hypothesised that this type of reaction is likely caused by "the existence of pathogen field population interactions with different host genotypes." They have shown that cultivar Erntestolz and Ditta have had varying symptom expression when inoculum originated from New Zealand, USA, Switzerland and Japan were used. The inoculum used in this study was from Tasmania (Australia), while the ranking used for analysis was based on the screening conducted in New Zealand and Europe. Nevertheless, Desiree, had a generally consistent reaction which was in between the very resistant (Gladiator) and very susceptible (Iwa) potato cultivars. This translates that Desiree has intermediate or moderate response to *S. subterranea* both in zoospore settlement (this study) and in the field (Falloon *et al.*, 2003, Merz *et al.*, 2012).

In conclusion, this study indicates that *S. subterranea* zoospore encystment is inhibited in resistant potato cultivars. Since the inhibition was strongly correlated with resistant ratings, inhibition of zoospore encystment is therefore an expression of cultivar resistance to *S. subterranea.* Furthermore, the bioassay method described here allows the assessment of resistance based on the number of zoospores encysted. Using very susceptible and very resistance cultivars as standards, the bioassay described in this study may be useful in rapidly screening potato lines or clones for resistance to *S. subterranea* root infection.